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EFFECT OF PARTICULATES ON DISINFECTION OF ENTEROVIRUSES IN WATER BY CHLORAMINES

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by

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MUNICIPAL ENVIRONMENTAL RESEARCH LABORATORY
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16. ABSTRACT

The inactivation kinetics of chloramines (monochloramine, NH_2Cl, and dichloramine, $NHCl_2$) on single preparation of an enterovirus, poliovirus 1 (Mahoney), and an enteric indicator of fecal pollution, Escherichia coli 11229, were examined in closely controlled laboratory studies using the kinetic (stirred beaker) apparatus. The disinfecting ability of chloramines effected by particulates (HEp-2 and BGM cell-associated virus preparations, feces-solids, and primary effluent-solids was determined. Additionally, comparisons were made between chloramines and other disinfectants (i.e. hypochlorous acid, HOCl, and hypochlorite ion, OCl^-) regarding aggregated vs. single virus preparations, different temperatures of reactivity, different pH's, and different disinfectant combinations. Aggregated poliovirus inocula were about 1.7 times more resistant to NH_2Cl than the singles preparation. Doubling the NH_2Cl dose from 12 to 22 ppm did not double the rate of virus disinfection. A comparison between virus singles NH_2Cl disinfection rates at pH 7.0 and 9.0 showed no difference, but NH_2Cl at pH 7.0 was found to be a more effective disinfectant for E. coli than at pH 9.0. Forming NH_2Cl disinfected at a more rapid rate than performed NH_2Cl. Repeated exposure of poliovirus to NH_2Cl apparent increased resistance to inactivation. Protection of HEp-2 cell-associated poliovirus when disinfected with NH_2Cl occurred, but no differences in the disinfection rates occurred when BGM cell-associated poliovirus was disinfected by $NHCl_2$.

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FOREWORD

The Environmental Protection Agency was created because of increasing public and government concern about the dangers of pollution to the health and welfare of the American people. The complexity of that environment and the interplay between its components require a concentrated and integrated attack on the problem.

Research and development is that necessary first step in problem solution and it involves defining the problem, measuring its impact, and searching for solutions. The Municipal Environmental Research Laboratory develops new and improved technology and systems for the prevention, treatment, and management of wastewater and solid and hazardous waste pollutant discharges from municipal and community sources, for the preservation and treatment of public drinking water supplies, and to minimize the adverse economic, social, health, and aesthetic effects of pollution. This publication is one of the products of that research; a most vital communications link between the researcher and the user community.

This research provides basic and pertinent information concerning the viral disinfection capabilities of chloramines as compared to free chlorine forms, and the effect of viral aggregations, and organic turbidities in water on the effectiveness of chloramines against enteric viruses.

Francis T. Mayo, Director
Municipal Environmental
Research Laboratory

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ABSTRACT

The inactivation kinetics of chloramines (monochloramine and dichloramine) on an enterovirus, poliovirus 1 (Mahoney) and an enteric indicator of fecal pollution, Escherichia coli 11229, were examined in laboratory bench-scale studies using the kinetic (stirred beaker) apparatus. The disinfecting ability of combined chlorine forms of chloramines as affected by viral aggregates and organic particulates was compared to viral inactivation in pure buffer systems with unassociated viruses and without added particulates. Additionally, comparisons were made between chloramines, hypochlorous acid and hypochlorite ion in a variety of different test situations, such as (1) the type of particulates (enterovirus-associated animal cells, solids-associated primary effluents and fecal suspensions), (2) aggregated versus unassociated single viruses, (3) different temperatures of reactivity, (4) different pH's, and (5) different disinfectant combinations.

Comparison of the relative inactivation rates at pH 9 and 15C at the 99% inactivation level showed that the aggregated poliovirus preparation was 1.7 times more resistant to monochloramine than the unassociated singles preparation of poliovirus. Plots of 99% inactivation data for monochloramine disinfection showed extended tailing for the individual survival curves, whereas the 90% inactivation data provided equally spaced concentration-time relationships at pH 9 and at temperatures of 5, 15, and 25C. Almost doubling the monochloramine dose at 5C and pH 9 from 12 mg/L to 22 mg/L did not double the rate of virus disinfection.

Dichloramine was less effective in the inactivation of poliovirus 1 than monochloramine. The single viral preparation was inactivated by dichloramine at the 90% level about eight times as rapidly as the aggregated inoculum. Using 90% inactivation by dichloramine of poliovirus 1 singles at 5 and 15C, a 10-degree increase in temperature gave a Q_{10} of 2.5.

Monochloramine was subsequently formed at pH 9 and then adjusted to pH 7 to give a stable solution of mostly monochloramines. Monochloramine disinfection rates were then examined at the two different pH's. Monochloramine disinfection rates of single virus particles at pH 7 and 9 showed no difference, however for E. coli at pH 7 monochloramine was a more effective disinfectant than at pH 9. The rate of disinfection of E. coli at pH 7 with 2.0 mg/L monochloramine was about ten times as rapid as that at pH 9 with 2.2 mg/L monochloramine.

Comparison of the disinfection of E. coli using newly formed preformed and forming monochloramine was made. The forming monochloramine was about 1.2 times more effective than the preformed monochloramine at 5C and pH 9. Sequential addition of poliovirus 1 after inital exposure of poliovirus 1

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singles to monochloramine resulted in a reappearance of the initial inactivation rate, indicating that the monochloramine had not been altered or destroyed.

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Disinfection of HEp-2 cell-associated poliovirus with 2.28 mg/L hypochlorous acid at 5C and pH 6 was 40 times more effective than 12.2 mg/L monochloramine at 5C and pH 9. Increasing the monochloramine dosage from 12.2 to 21 mg/L at similar turbidities (1.65 NTU and 1.5 NTU, respectively) reduced the time required for 90% inactivation, from 50 minutes at 12.2 mg/L to 30 minutes at 21.0 mg/L. At pH 7, increasing turbidities to 20 NTU from 1.5 at almost the same monochloramine levels (i.e. 11.30 and 10.35 mg/L, respectively) decreased disinfection efficiency.

Comparisons between BGM cell-associated poliovirus disinfection with hypochlorous acid (1.20 mg/L at 15C, pH 6 and 1.1 NTU), and monochloramine (1.16 mg/L at 15C, pH 9 and 1.6 NTU) showed that under these conditions hypochlorous acid is 380 times as effective as monochloramine. When BGM cell-associated poliovirus 1 and unassociated single virions were disinfected with dichloramine at 5C and pH 4.5, no differences in disinfection rates were observed. Changing the pH of monochloramine from 9 to 7 had no apparent effect on the disinfection rate of unassociated poliovirus 1 singles or cell-associated viruses.

Disinfection studies of total coliforms in fecal suspensions using 0.71 mg/L hypochlorous acid (final level of 0.27 mg/L) at 5C, pH 6 and 3.2 NTU showed an inital rapid die-away of more than 99.9% during the first minute, then the curve became asymptotic for the next 5 minutes. Protection of naturally-occurring coliforms found in primary effluents after disinfection with monochloramine (5.1 to 23.2 mg/L) at 5C and pH 7 occurred at various turbidities (1.8 to 8.0 NTU).

Survivors of poliovirus 1 that had been exposed 8 times at 15C and pH 9 to monochloramine were subsequently disinfected with 8.95 mg/L of monochloramine also under the same conditions. These survivors were now 2.3 times more resistant than the initially unexposed virus.

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SECTION 1

INTRODUCTION

OBJECTIVES

The objectives of this concluded research study were multiple, but consistent with EPA's goal of providing virus-free water to the consumer that contain low or no levels of suspect carcinogens. The main objective investigated was the effects of particulates in water on enterovirus-disinfection with chloramines as the disinfectant. The complete study objectives can be summarized thusly:

a. To determine the effect of water containing particulates on the disinfection of test microbes (primarily poliovirus 1 and the reference bacterium Escherichia coli) using combined available chlorine (the chloramines). These results were then compared to those of free chlorine (hypochlorous acid and hypochlorite ion). Particulate material was then examined as to its effect on disinfection efficiency, and included human fecal solids, sewage-primary effluent solids, and animal cell associated-poliovirus 1.

b. The disinfection ability of chloramines (both monochloramine and dichloramine) was studied at various chloramine levels, temperatures, contact times, and pH values; concentrations and types of particulates; and single versus aggregated preparations of test virions. Comparisons were also made of the disinfection efficiencies of monochloramine used as a preformed dose to that of forming doses of monochloramine. The effect of doubling the monochloramine dose upon virus inactivation, and the addition of multiple doses of poliovirus 1 during the progress of the experiment were also studied.

c. The selection of a monochloramine-resistant poliovirus 1 mutant.

All of the disinfection studies were performed using the kinetic apparatus of Scarpino et al. (1,2).

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BACKGROUND

Chloramines and Trihalomethane Formation

The use of combined chlorine (i.e., chloramines) for disinfection of water supplies assumes great importance when consideration is given to their reduced reactivity (compared to chlorine) with precursor organics in water to form potential carcinogenic trihalomethanes (THMs) (3). Investigations have shown that chloramines either do not cause trihalomethane (i.e., the most common types are chloroform, bromodichloromethane, dibromochloromethane, and bromoform) formation, or cause reduced quantities of trihalomethanes to be formed.[4] Based upon the potential dangers associated with THMs, the U.S. Environmental Protection Agency added in 1979 a maximum contaminant level (MCL) for THMs to the Safe Drinking Water Act. This standard included only the four most common THMs types listed above, and the MCL for them was set at 0.10 mg/L (i.e., 100 µg/L).

The 1975 National Organics Reconnaissance Survey (NORS) (5) initially had noted that the finished water of utilities disinfecting with chloramines contained total trihalomethane (TTHM) levels that ranged from 1 to 81µ g/L (19 µg/L average), whereas those water utilities using breakpoint chlorination had TTHM concentrations ranging from 1 to 472 µg/L in their finished water, with an average level of 72 µg/L. The reason why some of the water utilities in the NORS study using chloramines had high TTHM was probably due to free chlorine being used prior to the addition of ammonia, with the resultant formation of TTHM in the water (4). Subsequently, Stevens et al. (6) added both free and combined chlorine for varying contact times to untreated Ohio River water. Their results are presented in Figure 1.

The TTHM level in the free chlorine dosed sample reached 160µ g/L after 72 hours, whereas that sample dosed with combined chlorine (i.e., chloramines) formed only 16 µg/1 TTHM during the same time period. Thus, the toxicological hazard associated with the use of the alternative disinfectant, the chloramines, is lessened in regard to the THMs. One study (7) indicated, however, that monochloramine (NH_2Cl) was mutagenic in Bacillus subtilis, whereas results from an initial 90-day carcinogenic bioassay study using NH_2Cl exposed mice showed in their livers increased mitotic figures, bizarre chromatin patterns, and increased cell size (8). On the other hand, hemolytic anemia has not been found in animals orally consuming levels as high as 100 mg/L of NH_2Cl. Obviously, more lifetime animal studies will be required in order to assess fully the toxicological hazard of chloramines in drinking water. It is also necessary to conduct further studies regarding chloramine disinfection capability in regard to turbidities and microbes of pathogenic significance in water supplies, especially the animal viruses (4,9). This present study was thus primarily concerned with the virus-kill efficiency of the chloramines as alternative water disinfectants, singly and in the presence of natural or simulated turbidities.

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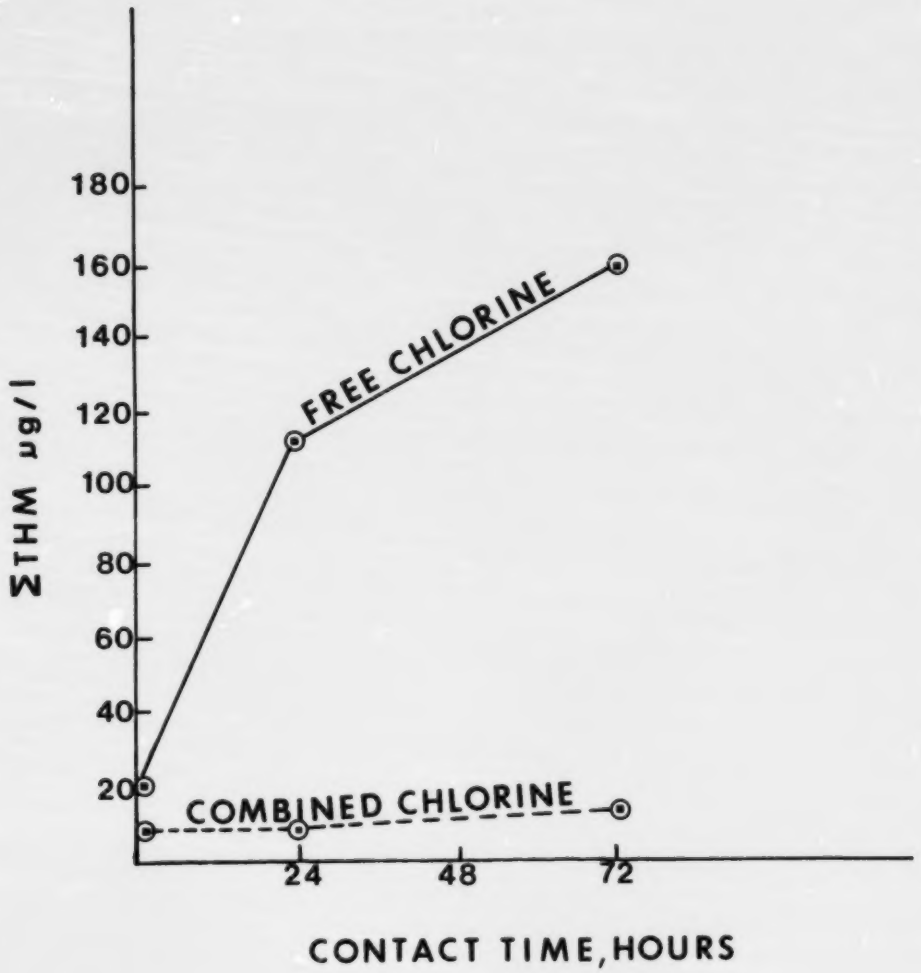

Figure 1. The formation of trihalomethanes in Ohio River water after the addition of free and combined chlorine (6)

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Human Enteric Viruses In Water

Precise knowledge concerning the inactivation of viruses in water assumes greater importance as man turns to an ever increasing degree to the re-use of his upstream neighbor's wastewater. Since sewage-contaminated water is a potential health hazard (10), an awareness of the efficiency of applied disinfectants such as chloramine on human enteric pathogens has increased significance. This is particularly true with the viruses, which are considerably more resistant to disinfection than the bacteria. Over 100 new human enteric viruses have been described since the investigations of Enders et al. (11) on viral propagation techniques using tissue cultures. Enteric viruses infective for man are the most important viral agents known to be present in water and wastewater, and more than 100 different types may be expected in human feces; these are listed in Table 1 along with their associated diseases (12). Thus, the enteric viruses consist of the enteroviruses (primarily polioviruses, coxsackieviruses, and echoviruses), hepatitis type A, Norwalk type agents, rotaviruses, reoviruses, adenoviruses, and parvoviruses. Other viruses may be swallowed by humans (e.g., influenza, mumps, and cold or fever sore viruses), and may also be later isolated from our feces. However, these latter are not believed to be particularly significant in disease transfer via contaminated water. Clarke et al. (13) pointed out that since enteric viruses are found in the feces of infected individuals and are readily isolated from urban sewage, especially in the late summer or early fall, they may enter water supplies and present health hazards to humans. However, it was noted that the number of recognized water-borne outbreaks of enteric virus disease was not large, which indicated that many outbreaks may not be reported or understood to be viral in origin.

Virologists in a number of countries have now reported the presence of enteroviruses in drinking water samples obtained from public water supply systems, including those systems that treat the water by conventionally accepted methods of filtration followed by disinfection (14-19). More recently, Sekla et al. (20) and Payment (21) reported the isolation of viruses from drinking water. Payment's study is particularly important because of his consistent (i.e., from every sample tested) and high (i.e., most samples contained 1-10 cytopathogenic units/100 liters of drinking water) recoveries of viruses from finished drinking water leaving a water treatment plant that practiced prechlorination, flocculation, sand filtration, and ozonation, followed by postchlorination. A residual free chlorine level of about 0.2 to 0.3 mg/L at pH 7.5 was maintained throughout this study. This residual, however, was below the recommended free chlorine residual of 0.5 mg/L (maintained for a contact time of 30-60 minutes) as recommended by the World Health Organization (12). Bacteriologically the finished water was safe, since all the samples were negative for coliforms. However, poliovirus 1 was a frequent isolate, but many isolates were non-polioviruses. Payment (21) pointed out that all the studies that have reported the presence of viruses in finished water share the commonality of being bacteriologically safe and adequately disinfected with a residual chlorine level considered to be virucidal. It was speculated that the passage of the viruses through the plant's treatment train could be due to one or more of the following: an enhanced viral resistance to chlorine, as reported by Bates et al.(22); the presence

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TABLE 1. Human Enteric Viruses that may be Present in Water and their Associated Diseases (12)

Virus group	No. of types	Disease caused
Enteroviruses:		
Poliovirus	3	Paralysis, meningitis, fever
Echovirus	34	Meningitis, respiratory disease, rash, diarrhoea, fever
Coxsackievirus A	24	Herpangina, respiratory disease, meningitis, fever
Coxsackievirus B	6	Myocarditis, congenital heart anomalies, rash, fever, meningitis, respiratory disease, pleurodynia
New enteroviruses	4	Meningitis, encephalitis, respiratory disease, acute haemorrhagic conjunctivitis, fever
Hepatitis type A (probably an enterovirus)	1	Infectious hepatitis
Gastroenteritis virus (Norwalk type agents)	2	Epidemic vomiting and diarrhoea, fever
Rotavirus (Reoviridae family)	?	Epidemic vomiting and diarrhoea, chiefly of children
Reovirus	3	Not clearly established
Adenovirus	30	Respiratory disease, eye infections
Parvovirus (adeno-associated virus)	3	Associated with respiratory disease in children, but etiology not clearly established

Note: Other viruses which, because of their stability, might contaminate water are the following:

(1) SV-40 like papovaviruses, which appear in the urine. The JC subtype is associated with progressive multifocal leukoencephalopathy.

(2) Creutzfeld-Jakob (C-J) disease virus. Like scrapie virus, the C-J virus resist heat and formaldehyde. It causes a spongiform encephalopathy, characterized by severe progressive dementia and ataxia.

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of natural particulate matter; the association of the viruses with the alum used for flocculation; or more probably by virus association with organic matter (23). In any case these reports on the isolation of viruses from the treated drinking water appear to be at variance with the statement of the AWWA Committee on Viruses in Drinking Water (24) that optimal and consistently-applied full water treatment (i.e., coagulation-sedimentation and filtration steps followed by disinfection, usually with chlorine as in the United States) will provide reasonable assurance of a virologically safe finished drinking water. Obviously, enteric viruses are being isolated from samples of supposedly finished drinking water considered safe for drinking; concern must therefore be expressed at the possible health risk posed by these viral isolates. Additionally, if water utilities turn to the use of chloramines as a water disinfectant because of their lessened effect on the formation in drinking water of potentially carcinogenic chlorinated organic compounds, more precise information must be forthcoming as to chloramine disinfection efficiency alone and in the presence of particulate matter in drinking water (i.e., high turbidities).

Low Level Transmission of Viruses

Epidemiologically, low level transmission of viruses to man is important when consideration is given to what constitutes a minimum oral virus dose capable of producing infection and disease in man. Berg (25) stated that the ingestion of small quantities of viruses by relatively small numbers of people daily would result in disseminated illnesses that would produce an epidemiologic picture consistent with person-to-person transmission. This situation would more likely produce asymptomatic carriers, making it difficult to indict the water route. This view has been challenged since no evidence in support of the low-level transmission hypothesis has yet been presented (26). However, available data does show that the minimum infective dose of enteroviruses to man is low, as seen in Table 2. Plotkin and Katz (27) reviewed the available literature concerned with the minimum virus dose infective for man by the oral route. Experimentation by these workers with attenuated poliovirus demonstrated that one tissue culture unit (1 $TCID_{50}$) could constitute an infectious dose (28). Animal studies by Westwood and Sattar (29) supported the conclusion of Katz and Plotkin. These studies and others reported by Westwood and Sattar suggested a near-parity in the cell-infective doses. The 1981 studies with human volunteers by Stafanovic et al (34) indicated that 10 plaque-forming units (PFU) of an enterovirus (i.e., the ECHO-12 virus) ingested in drinking water resulted in human infections. Additionally, the infection resulted in a shedding state for as long as 19 days at dosages as low as 10 PFU. An IAWPRC study group on water virology concluded that based upon available data, although "the minimal infectious dose of enteric viruses is generally in considerable excess of 1 PFU, there is reason to believe that certain highly susceptible individuals may indeed be infected by a single PFU." (26).

Infectivity of Particulate-Associated Viruses

The majority of viruses in the natural environment are associated with solids and are not in a "free" state (35). Wastewater influent, effluent, and chlorinated effluent samples were found to have 16.1 to 100% of their

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TABLE 2. Oral Infective Dose to Man of Enteric Viruses

Virus	Human Subjects	Dose	Method Of Administration	Percent Infected	Reference
Poliovirus 1 (Attenuated SM stain)	Adults	0.2 PFU[a]	Gelatin capsule	0	Kaprowski, 1955/56 (30,31)
		2.0 PFU		67	
	Infants	20 PFU		100	Kaprowski et al., 1956 (32)
		200 PFU		100	
Poliovirus 3 (Attenuated Fox strain)	Premature infants	1.0 $TCID_{50}$[b]	Gavage tube	30	Katz and Plotkin, 1967 (28)
		2.5 $TCID_{50}$		33	
		10.0 $TCID_{50}$		67	
Poliovirus 1 (Sabin strain)	Infants	16	Aqueous suspension	0	Minor et al., 1981 (33)
		50		50	
		90		75	
		160		100	
Echovirus 12 (Wild strain)	Young male adults	10	Aqueous suspension	19	Stephanovic et al., 1981 (34)
		30		29	
		100		67	

[a]Given as plaque-forming units (PFU)

[b]Given as the quantity of virus that will infect 50% of the tissue cultures inoculated ($TCID_{50}$)

total virus content associated with solids (36). The association of viruses with solids does not necessarily mean virus inactivation; in fact, clay solids do not appear to have any deleterious effect on the viruses. Moore et al. (37) presented data that reaffirmed the findings of others (38-40) that viruses associated with suspended particulates were infective, by finding that most of their test enteroviruses were infective by plaque assay in their particulate-adsorbed form. Thus, monitoring of environmental virus levels must account for not only free virus but also for those that are solids-associated.

The concern that particulates (causing turbidity) in drinking water may also interfere with the disinfection process is well-founded. Clarke and Chang (41) believed that turbidity caused by particulates was responsible for the disinfection failure that resulted in the Delhi, India, infectious hepatitis outbreak of 1955. Neefe et al. (42) reported that feces from an infectious hepatitis patient after suspension in water and treatment with chlorine to a final residual of 1.1 mg/L for 30 minutes still caused hepatitis in two of five human volunteers. Walton (43) detected coliform bacteria in chlorinated water from a water-works that usually did not have turbidities greater than 10 Turbidity Units (TU). Robeck et al. (44) demonstrated in a pilot-plant seeded with viruses that virus attached to flocs could penetrate a granular filter and be recovered from the effluent with as little as a 0.5 TU increase in turbidity. Sanderson and Kelly (45) recovered coliform organisms from household taps with water that received no treatment other than chlorination and had turbidities that varied from 4 to 84 TU. Tracy et al. (46) recovered coliform bacteria in chlorinated unfiltered water supply with turbidities of from 5 to 10 TU. Studies on the effects of inorganic turbidity on the disinfection of poliovirus 1 adsorbed onto bentonite clay, and poliovirus 1 precipitated by aluminum phosphate ($AlPO_4$) were reported by Symons and Hoff (47). They observed similar disinfection curves for the poliovirus adsorbed to bentonite or $AlPO_4$ at about 5 Nephelometric Turbidity Units (NTU) and 1.5 mg/L HOCl. No indication of a protective effect was found when these survival curves were compared to unassociated poliovirus controls. Stagg et al. (48) reported the inactivation of bentonite-adsorbed bacteriophage MS-2 with HOCl. At equivalent HOCl concentrations ranging from 0.02 to 0.6 mg/L, approximately twice the time was required for 99% inactivation of the bentonite-adsorbed bacteriophage MS-2 as for free-associated virus at turbidities that varied from 2 to 4 Jackson Turbidity Units (JTU). Boardman and Sproul (49) reported that when bacteriophage T_7 was adsorbed to either calcium carbonate, hydrated aluminum oxide, or kaolinite clay and exposed to chlorine at pH 7 at 22C no protection of adsorbed virus resulted. (The particulates were measured in mg/L, not as turbidity.) Gerba and Stagg (50) disputed the results of Boardman and Sproul based on the bacteriophages ultrastructure and size when compared to that of animal enteric viruses. They also disagreed with the experimental sampling method employed by the latter, arguing that short reaction times of less than 1 minute were necessary to detect protective effects. (Stagg et al. (48) used contact times of disinfectant and adsorbed-virus of less than 1 minute). Studies by Scarpino et al. (51) using ClO_2 as the disinfectant found a correlation between bentonite protection of poliovirus 1 during disinfection at increasing temperatures and increasing turbidities; i.e., as the temperature and bentonite

turbidity increased, the disinfection efficiency decreased for the bentonite-adsorbed poliovirus 1.

The effects of organic turbidity on the disinfection process have also been studied with cell-associated viruses. Cell-associated enteric viruses are found in domestic sewage, and are produced in cells that line the human intestinal tract and which eventually slough off, becoming part of the excreta that forms domestic sewage. Cell-associated viruses in source waters may therefore enter potable water treatment plants, and the viruses may then pass through the disinfection process in a viable state. Moffa et al. (52) reporting on the inactivation of cell-associated poliovirus 1 present in simulated combined sewer overflow water observed that the presence of cellular material interfered with the effectiveness of disinfection by chlorine dioxide. Symons and Hoff (47) reported that cell-associated poliovirus 1 with a turbidity of 1.4 Nephelometric Turbidity Units (NTU) was protected from inactivation by HOCl at pH 6 at 5 C. The initial HOCl residual for the cell-associated poliovirus was about 2.0 mg/L after 5 minutes of contact time. Free-unassociated poliovirus was reduced by 5 logs in less than 1 minute, while cell-associated poliovirus was reduced by about 3 logs in 5 minutes. Scarpino et al. (51) determined for ClO_2 inactivation of cell-associated poliovirus 1 versus unassociated poliovirus 1 that no trend was evident toward protection of the virus at the turbidity levels examined. This was believed due to the cell-associated poliovirus 1, existing in a "singles" or non-aggregation state, and that the cellular material was oxidized off the poliovirus by the ClO_2.

Waterborne animal viruses may be transported either inside or on the surface of cells that are excreted from the intestinal tract of man and animals. The possibility exists that under natural conditions these cell-associated viruses may be protected during disinfection. Of particular interest is the research dealing with minimal infectious dose. For instance, the turbidity quality of the drinking water may be crucial in altering the virus minimal infectious dose, since viruses in the water can be protected by turbidity-causing materials, and thus reach the human gastrointestinal tract more readily than unprotected virions. This possibility for viral and bacterial survival during the water treatment disinfection process led to the national interim primary drinking water regulations allowing a maximum contamination level of 1 NTU, with up to 5 NTU allowed if it could be demonstrated that the latter turbidity level did not interfere with disinfection, prevent maintenance of effective disinfection throughout the distribution system, or interfere with microbial determinations (53).

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SECTION 2

CONCLUSIONS

1. Virus aggregates are, along with organic particulates, a major part of the mechanism for the survival of virus infectivity in water. In these studies, aggregated poliovirus 1 (at the 99% inactivation point) at 15C and pH 9 was about 1.7 times more resistant to disinfection by monochloramine than unassociated virus singles. The singles virus preparation disinfected by dichloramine at 15C and pH 4.5 was inactivated (at the 90% inactivation point) about 8 times as rapidly as the aggregated virus.

2. An average Q_{10}* value of 2.75 was obtained in monochloramine-temperature reactivity studies with poliovirus 1 singles at pH 9 at temperatures of 5, 15, and 25C. For dichloramine, a 10-degree change in temperature gave a Q_{10} of 2.5 for poliovirus 1 singles. Both Q_{10} values are within the 2 to 3 factor increase noted by Clarke and Chang (41).

3. Monochloramine formed at pH 9 and then adjusted to pH 7 was a better disinfectant for bacteria but not for the test virus. Lowering the pH from 9 to 7 increased monochloramine disinfection efficiency about 10 times for the bacteria.

4. In the comparison of the disinfection of E. coli using preformed and forming monochloramines, the forming monochloramine was about 1.2 times more effective than the preformed monochloramine. The faster disinfection rate could be due to the initial presence of hypochlorous acid before the monochloramine was completely formed.

5. A gradual progression in the development of virus resistance to monochloramine was found with the Floyd et al. (56) inocula. Survivors of poliovirus 1 exposed 8 times to monochloramine and then disinfected with 8.95 mg/L monochloramine were 2.3 times more resistant to monochloramine than either monochloramine-unexposed virus or the virus previously exposed 7 times to monochloramine.

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* Q_{10} is a doubling of the reaction rate per a 10 degree increase in temperature within defined temperature ranges.

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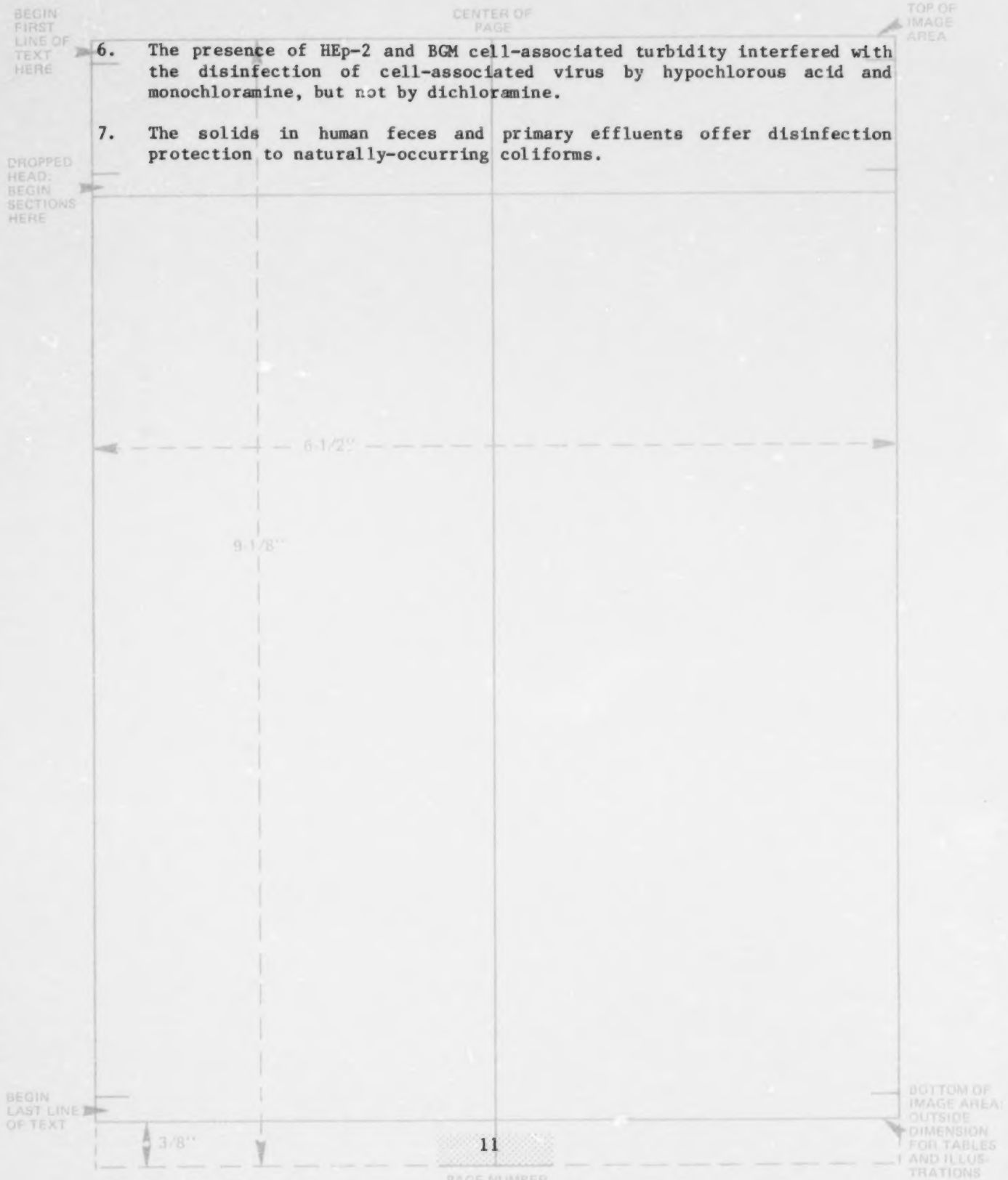
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6. The presence of HEp-2 and BGM cell-associated turbidity interfered with the disinfection of cell-associated virus by hypochlorous acid and monochloramine, but not by dichloramine.
7. The solids in human feces and primary effluents offer disinfection protection to naturally-occurring coliforms.
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SECTION 3

RECOMMENDATIONS

Since most finished drinking waters are maintained in the United States at a pH level below 9 the dramatic increase in monochloramine disinfection efficiency for E. coli by lowering the pH of monochloramine from 9 to 7 should be further investigated as to its possible mechanism of action. In addition, other animal viruses besides poliovirus should be studied to determine if they also are affected by the pH change.

Additional studies are required with different cell lines to determine if they have similar viral protective effects during disinfection.

Feasibility studies of the cost effectiveness of reducing the turbidity levels from 5 to 1 NTU in drinking water treatment should be determined. Implementation of a reduced turbidity level to 1 NTU is recommended because of our studies and those of others with coliforms associated with primary effluent solids, fecal solids and cell-associated viruses.

In future turbidity studies, ways of determining the nature of the particulates (inorganic or organic) must be developed to ascertain their potential for protection during disinfection.

Methodology guidelines/recommendations should be established as to disinfection experimental apparatus and the physical state of the test organisms, i.e., viral associated (aggregates or cell-associated) and unassociated (singles) preparations.

The usefulness of chloramines, especially the monochloramines, in field situations should be more carefully evaluated. Under certain definite conditions (such as "forming" situations) they may be useful.

Dichloramine's ability to penetrate organic masses, such as cells, should be more thoroughly investigated.

The development of resistant strains of viruses in nature should be thoroughly studied. Laboratory studies can only point out a potential problem; field studies are required to pin point possible health risks that might exist in the natural environment.

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SECTION 4

MATERIALS AND METHODS

PREPARATION OF CHLORINE SOLUTIONS

Free Available Chlorine

Stock solutions of chlorine were prepared as required by bubbling chlorine gas for 30 minutes into an amber bottle containing deionized distilled water held at a temperature of 5C and subsequently stored at the same temperature. Chlorine gas reacted with water to form hypochlorous acid (HOCl) and subsequently hypochlorite ion (OCl^-) according to the following reactions (Eq. 1 and 2):

$$Cl_2 + H_2O \rightleftharpoons HOCl + H^+ + Cl^- \qquad \text{Eq. 1}$$

$$HOCl \rightleftharpoons H^+ + OCl^- \qquad \text{Eq. 2}$$

The dissociation of hypochlorous acid is dependent chiefly upon pH and, to a much lesser extent, temperature, with almost 100% HOCl present at pH 5, and almost 100% OCl^- present at pH 10. Free available chlorine refers to the concentration of hypochlorous acid and hypochlorite ion, as well as any molecular chlorine existing in a chlorinated water. On the day of the tests using HOCl or OCl^-, the desired chlorine concentrations were prepared in demand-free buffer by the addition of a pre-calculated amount of the stock chlorine solution.

Combined Available Chlorine

Inorganic chloramines are formed by three successive substitution reactions between aqueous chlorine (i.e., hypochlorous acid) and ammonia (NH_3) in accord with the following equations:

$$HOCl + NH_3 \rightleftharpoons NH_2Cl + H_2O \qquad \text{Eq. 3}$$

$$HOCl + NH_2Cl \rightleftharpoons NHCl_2 + H_2O \qquad \text{Eq. 4}$$

$$HOCl + NHCl_2 \rightleftharpoons NCl_3 + H_2O \qquad \text{Eq. 5}$$

The proportion of chloramines formed, called monochloramine (Eq. 3), dichloramine (Eq. 4), and nitrogen trichloride (Eq. 5) depend chiefly upon the relative amounts of HOCl and NH_3, and the pH of the solutions. To a minor extent, the time of contact and temperature also affects the chloramine distribution. Dichloramine ($NHCl_2$) is the predominant form of chloramine at

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a 1:1 molar ratio of ammonia to chlorine at pH values of 5 and below, whereas at pH values of 9 and above, monochloramine (NH_2Cl) predominates. Figure 2 shows the proportion of monochloramine and dichloramine formed for pH values of 4 to 9 and temperatures of 0C, 10C, and 25C (54). Combined available chlorine refers to the concentration of inorganic chloramine. Since chlorine will also react with organic amines, the organic chloramines that are formed are also included in the term combined available chlorine.

In these studies, monochloramine was formed by first chlorinating the pH 9 borate buffer to the desired level, and then adding ammonium sulfate [$(NH_4)_2SO_4$] to produce an ammonia (NH_3) to chlorine ratio of 6:1 (by weight), as in Eq. 3. This meant 6 mg NH_3 to 1 mg titrable Cl_2, or 23 to 1 on a molar basis. There was no free chlorine present in the solution by amperometric titration. At pH 9 and at a 23 to 1 molar ratio of ammonia to chlorine, practically all titratable chlorine exists as monochloramine. At pH values greater than 8 only monochloramine is usually noted. Dichloramine was formed by adding ammonium sulfate to one to two liter quantities of hypochlorous acid in pH 4.5 demand-free 0.05 M phthalate buffer. The chlorine to nitrogen weight ratio (Cl:N) was 3:1 to insure rapid formation of the dichloramine. The solution, loosely capped, was allowed to mix on a Magnistir for two hours at room temperature to allow maximum dichloramine formation. Since dichloramine was found to be unstable over a 24-hour period, test solutions were freshly prepared on the day of the experiment.

Nitrogen trichloride has little or no disinfection capability and is of chief significance in water treatment because of the obnoxious qualities it imparts to water.

CHLORINE DETERMINATIONS

Free and Combined Available Chlorine

The amperometric titration method employing the Wallace and Tiernan Amperometric titrator, was used to determine free chlorine and monochloramine at the time of the experimentation (1,2). Dichloramine was determined by the FAS:DPD method according to Standard Methods (55) with one exception. Since the titrations are required to be carried out at a pH of 6.2-6.5, the pH effect of the phthalate-buffered dichloramine samples had to be overcome. To accomplish this, pH 6 phosphate buffer was added as necessary to bring the pH of the titration sample to the proper range. The total recommended titration volume of 100 ml was held constant, as was the added amount of the DPD color indicator. The amperometric titration method was also used for dichloramine determinations.

NEUTRALIZER

The neutralizer for free and combined chlorine solutions consisted of sufficient sodium thiosulfate to give a final concentration of 6 mg/L in the samples removed from the test and control beakers.

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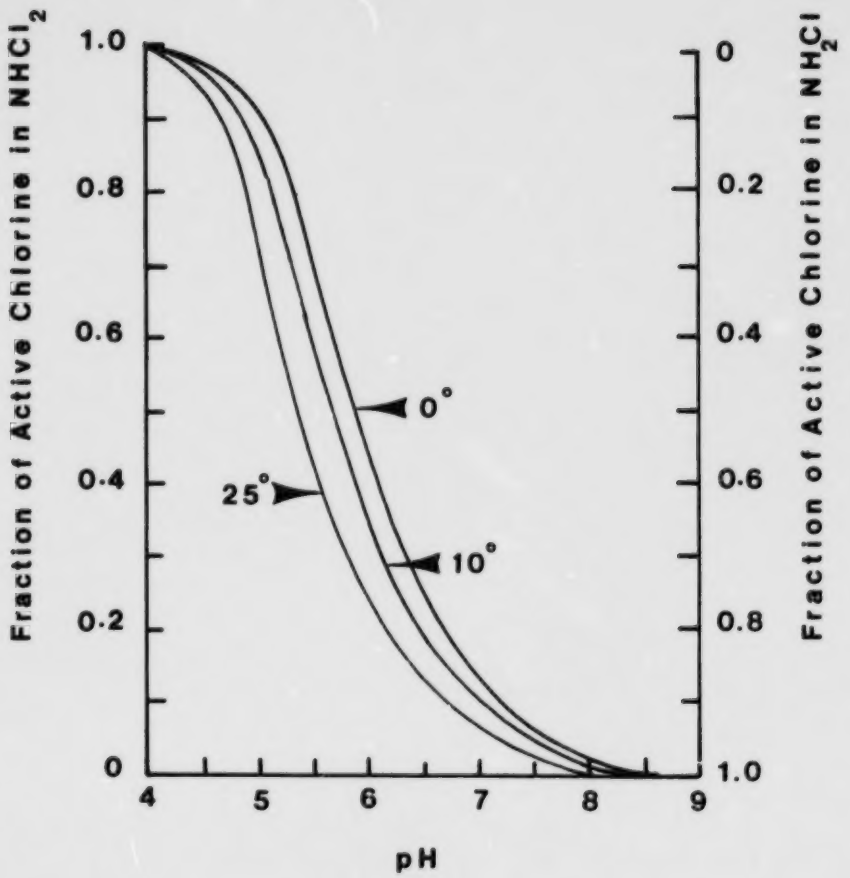

Figure 2. Proportions of mono- and dichloramine (NH_2Cl and $NHCl_2$) in water chlorination with equimolar concentrations of chlorine and ammonia (54)

PREPARATION OF BUFFER SOLUTIONS

Phosphate (KH_2PO_4-K_2HPO_4), borate (H_3BO_3-NaOH), and phthalate ($KHC_8H_4O_4$-NaOH) buffer systems at 0.05 M were used at various pH values to determine the effect of pH on the antiviral activity of free and combined available chlorine. The buffer system for the hypochlorous acid and hypochlorite ion studies consisted of 0.05 M, pH 6 phosphate buffer (KH_2PO_4-K_2HPO_4), and 0.05 M, pH 10 borate buffer (H_3BO_3-NaOH), respectively (1,2). For the monochloramine work, the test buffer was 0.05M borate (H_3BO_3-NaOH) at pH 9, or the above phosphate buffer at pH 7. For the dichloramine work, the test buffer was 0.05M potassium phthalate at pH 4.5. The buffers were made demand-free by the addition of the free available chlorine stock solution to provide a residual of 3 to 5 mg/L. After standing for several days, the water was dechlorinated at room temperature for at least 24 hours under ultraviolet light (chlorine is highly photoreactive at ultraviolet wavelengths of about 2600 Å and decomposes rapidly) until the DPD spot plate test method for chlorine was negative. The buffer was then considered to be chlorine demand-free.

PREPARATION AND PURIFICATION OF STOCK VIRUS

The poliovirus 1 (Mahoney strain) stocks in these studies were prepared in two different ways. In both methods the polioviruses were grown in monolayers of Buffalo Green Monkey (BGM) kidney continuous cell line obtained from Cercopithecus aethiops, the African Green monkey.

Aggregated Poliovirus Preparation

Poliovirus 1 was prepared from BGM cells which were infected approximately 24 hours earlier at a multiplicity of infection of 10 PFU per cell, and then incubated for one hour at 37C. MEM's maintenance media with 2% fetal calf serum was added to the infected tissue cultures and incubation was continued for 11 hours. When cytopathological effect (CPE) was apparent, the cells and fluids were collected and subjected to freezing (-70C) and thawing three times to release the virus particles contained in the cells.

The cell debris was spun out at 1,300 x g for 30 minutes, and the supernatant was spun at 128,000 x g for 3 hours. The pellet was suspended in chlorine demand-free water and spun to pellet the virus. This pellet was resuspended in chlorine demand-free water and tested for chlorine-demand. If none was present, the virus was diluted to 10^8 PFU/ml, placed into demand-free vials in 1 ml aliquots, and these vials were frozen at -70C. These virus preparations were referred to in this work as aggregated poliovirus preparations. If demand was present, the virus was resuspended in demand-free buffer and the precedure was repeated.

Preparation of Virus Singles

Purified poliovirus 1 inocula containing mostly single virions was prepared using the method of Floyd et al (56). BGM monolayers were inoculated with poliovirus 1 at a multiplicity of 100 PFU per cell. The virus was allowed to adsorb for 1 hour at 37C, after which the MEM's maintenance medium

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was added and the tissue culture cells were further incubated at 37C for 11 hours. The cells were then removed from incubation and chilled to 4C. The maintenance medium was now separately collected and centrifuged at 250 x g for 10 minutes to harvest the cells remaining in the medium itself. The chilled cells remaining in the tissue culture containers were then washed twice with phosphate buffered saline. The BGM monolayers were then scraped from the bottles, harvested by centrifugation (250 x g for 10 minutes) and pooled with the cells collected from the maintenance medium. The combined BGM cells were resuspended with phosphate buffered saline (6 ml) and the virus extracted by the addition of Freon 113 (4 ml) followed by homogenization (2 minutes) in a Waring blender, with separation of the virus containing aqueous phase from the freon phase by centrifugation (800 x g for 10 minutes). This freon extraction was carried out three times, each time the Freon phase was re-extracted with another 6 ml of phosphate buffered saline; and the phases were again separated. The upper virus-containing aqueous phases were collected, held in an ice bath, and made up to a final volume of 20 ml. The virus was now further purified and concentrated by density gradient centrifugation. The aqueous-virus phase was layered onto a 10% to 30% (wt/wt) sucrose gradient made with 0.05 M phosphate buffer at pH 7.2. The gradient was centrifuged at 80,000 x g in a Beckman L2-65B ultracentrifuge for 2.25 hours at 4C. Fractions of 2 ml each were collected from the centrifuge tubes and examined by sucrose refraction. Singles viruses appeared in the fractions containing 18-22% sucrose. All relevant fractions were pooled and stored at 4C without any attempt to remove the sucrose. The greatest concentration of purified virus was found at a concentration of 22% sucrose. The resultant virus preparation contained no cell debris, or debris of any kind, and consisted of greater than 93% single virus particles (51). This preparation was called poliovirus singles.

SELECTION OF MONOCHLORAMINE-RESISTANT POLIOVIRUS

Both the Bates et al. (22) and Floyd et al. (56) procedures for preparing the inocula used in their studies on selection of disinfectant-resistant poliovirus was utilized in our experimentation. The procedures used for preparation of the virus inocula are outlined in Figure 3.

PREPARATION OF CELL-ASSOCIATED VIRUSES

Enterovirus-associated animal cells were prepared to simulate naturally found cell-associated viruses which can be excreted from the intestinal tract of man. Two cell lines were used, i.e., HEp-2 (Human Epidermoid Carcinoma) and BGM cells. The HEp-2 cell-associated viruses were prepared by Dr. J. Hoff of MERL, U.S. EPA (57). Monolayer cultures were infected with poliovirus 1 at a multiplicity of infection of 5 PFU/cell. Infected cells were harvested, washed 6 times with chlorine demand-free 0.05M phosphate buffer to remove chlorine-demand, and titered. The total quantity of viruses associated only with the cells and cell debris was determined by first centrifuging the cell-associated virus preparation, and determining viral presence in the supernatant. The total amount of virus present which was associated only with the cell and cell debris was then determined by lysing the pelleted infected cells and cell debris with chloroform, thus releasing the associated viruses, and determining the total released viral titer.

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BGM cell sheets were inoculated with poliovirus 1 (Mahoney) at a multiplicity of infection of 100 PFU/cell. The virus was adsorbed for one hour at 37C. MEM's (containing 20% fetal calf serum) was added, and the cultures were incubated for 11 hours at 37C until CPE was observed.

↓

Follow Protocol For

↙ Bates et al. (22)

- Freeze and Thaw Fluids and Cells 3 Times
- Centrifuge at 10,000 x g for 20 Minutes to Remove Cell Debris
- Centrifuge Supernatant Fluids at 135,000 x g for 3 Hours
- Resuspend Pelleted Virus in 1.8 ml of Phosphate-Buffered Saline
- Disperse Virus by Sonic Oscillation
- Pass Virus through a 0.45 m Cellulose Nitrate Membrane Pretreated with 5 ml of a 1:5 Dilution of Fetal Calf Serum in Phosphate-Buffered Saline
- After Filtration, 0.2 ml of Fetal Calf Serum was Added to the Virus Sample to Give a Final Volume of 2 ml
- Determine Virus Titer by Plaque Assa

↘ Floyd et al. (56)

- Wash Cells with Phosphate-Buffered Saline and Scrap off Cells
- Extract Cells with Freon 113 in Blender for 2 Minutes
- Centrifuge to Separate Layers
- Extract 3 Times, Removing Aqueous Virus Layer Each Time
- Layer Aqueous Virus Layer onto 10 to 30% (wt/wt) Sucrose Gradien
- Centrifuge 80,000 x g for 2.25 Hours.
- Collect 2 ml Fractions
- Determine Sucrose % of Fractions By Refraction
- Pool 18-22% Fractions Containing Singles of Poliovirus 1
- Determine Virus Titer by Plaque Assay

Figure 3. Protocol for preparation of virus inocula used in cyclic exposure of poliovirus 1 to monochloramine

BGM cell-associated poliovirus 1 inocula were prepared by inoculating cell monolayers at a multiplicity of 100 PFU/ml. The virus was allowed to adsorb one hour at 37C prior to addition of MEM maintenance medium, and the cells were further incubated at 37C for 11 hours. The infected cells were then chilled to 4C. The monolayers were then scraped from the bottles, and they and their fluids were spun at 1,300 x g. The BGM cell-associated poliovirus 1 complex was then washed and spun 6 times with chlorine-demand-free 0.05 M phosphate buffer to remove chlorine-demand due to the animal cell presence.

PREPARATION OF SOLIDS-ASSOCIATED PRIMARY EFFLUENT COLIFORMS (58)

Five gallons of the City of Cincinnati's Little Miami Sewage Treatment Plant's unchlorinated primary effluent was passed through 90, 45 and 38 μm sieves. The collected solids were washed from the 38 μm sieve with 250 ml of pH 7, 0.05 M phosphate buffer. The solids were then washed, centrifuged for 15-20 minutes at 1,700 x g, and the pelleted solids were suspended in the buffer (100 ml volume/wash). This procedure was repeated 3 times to remove soluble chlorine-demand substances.

PREPARATION OF FECAL PARTICULATES

One gram of human fecal material was suspended in 100 ml chlorine demand-free pH 7, 0.05 M phosphate buffer by homogenizing the fecal material in a Sorval Omni-Mixer for 0.5 minutes at 11,500 RPM. The suspension was allowed to settle overnight, then the supernatant was centrifuged at 2300 x g the pellet was washed 3 times in the pH 7, 0.05M phosphate buffer, the pellet was resuspended in a small quantity of the demand-free phosphate buffer and was now ready for use.

PREPARATION OF BACTERIA

Escherichia coli (ATCC 11229) was grown in trypticase soy broth for 16-18 hours at 35C, centrifuged for 20 minutes at 2300 x g, and then the pellet was resuspended in chlorine-demand-free pH 7, 0.05 M phosphate buffer. The buffer washings of the cells were repeated 3 times. The final suspension was adjusted to an optical density (using a Klett-Summerson photoelectric colorimeter) equal to a cell concentration of 10^8 bacteria/ml. The suspension was usually found to be chlorine-demand-free. When necessary the cells were rewashed with demand-free phosphate buffer until the demand-free state was obtained.

MICROBIAL ASSAYS

Viral Assay:

Animal viruses were titered by the plaque technique of Dulbecco and Vogt (59), as modified by Hsiung and Melnick (60), in a continuous cell line, BGM, derived from primary African Green Monkey kidney cells. The BGM cell line has been found to be more sensitive to many enteroviruses (61). Most tissue cultures were prepared jointly by us and the Virology Section of the Biological Methods Branch, EMSL, U.S. Environmental Protection Agency, Cincinnati,

but BGM cell-associated poliovirus 1 inocula were prepared by us. HEp-2 (Human Epidermoid Carcinoma) cell cultures for the poliovirus 1 cell-associated studies were supplied by Dr. John Hoff of the U.S. Environmental Protection Agency, MERL.

Bacterial Assay

E. coli survivors in these studies were recovered and enumerated using surface-inoculated trypticase soy agar plates, or, for primary effluent work, using the Most Probable Number multiple-tube fermentation technique through the confirmed test (55). The plates were incubated for 24 hours at 37C prior to being counted.

TURBIDITY MEASUREMENT

Turbidity was measured in Nephelometric Turbidity Units (NTU) using a Hach 2100A Turbidimeter. The instrument was always standardized before use using turbidity standards obtained from the Hach Co., Loveland, Colorado 80539.

EXPERIMENTAL PROCEDURE

On the day of the experimentation, the desired free or combined chlorine concentrations were prepared in chlorine demand-free buffer by the addition of a calculated amount of the stock chlorine solution. The procedures outlined in (1) Preparation of Chlorine Solution and (2) Chlorine Determination above were used to prepare and analyze for the free and combined chlorine used in these studies.

Four hundred ml volumes of the disinfectant-treated buffer were then placed in 600 ml test beakers according to the test scheme used by Scarpino *et al*. (1,2), (Figure 4). These beakers were covered by a loose metal lid through the center of which passed a glass stirring rod. One buffer control beaker contained 400 ml of the untreated (no disinfectant was added) demand-free buffer, while a second neutralizer control beaker contained a treated neutralized buffer (containing the highest free chlorine, monochloramine or dichloramine concentrations being used that day). The chlorine species in the second beaker was neutralized with sodium thiosulfate just before the start of the experiment. Disinfectant control beakers were prepared that were similar in every aspect to the test beakers except that the test virus or bacterium was not added. The glass stirring rods were connected to an overhead variable-speed device that was adjusted to 81 RPM, and all beakers were allowed to equilibrate to the test temperature in a carefully regulated water bath. After temperature equilibrium was obtained, the disinfectant control beakers were titrated at the start (and end) of each experiment, whereas the test beakers were titrated at the end of each study. One ml standardized amounts of the test virus or bacteria were added to the buffer control, neutralizer control, and the test beakers; 5 ml samples were removed at intervals and rapidly added into 5 ml of the neutralizer. The neutralized samples were assayed immediately following the conclusion of the test. The pH of the test and control solutions were also determined at this time.

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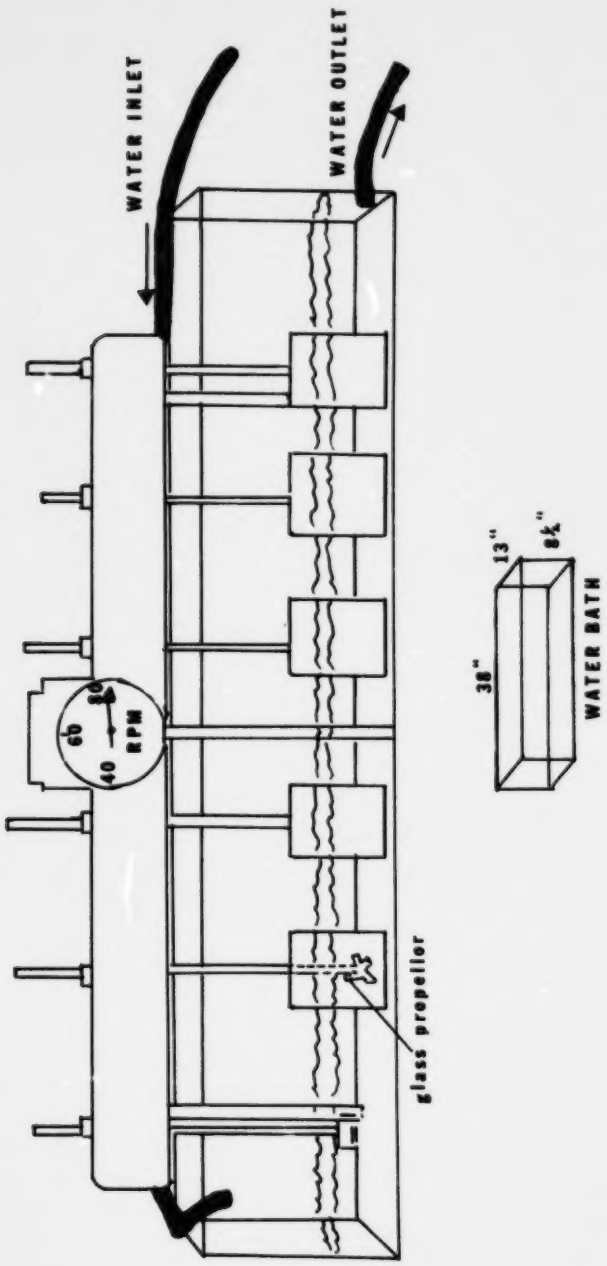

Figure 4. Kinetic (stirred beaker) apparatus (1,2)

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SECTION 5

RESULTS AND DISCUSSION

MONOCHLORAMINE DISINFECTION OF POLIOVIRUS 1 SINGLES AND AGGREGATES

A number of viral aggregation studies have implicated aggregates in the viral inocula as the cause of aberrations in survival curves when viruses are exposed to destructive chemical and physical agents, such as disinfectants (62,63). Scarpino et al. (64) concluded that their observed variations in chlorine dioxide disinfection survival rates occurred due to viral aggregation. In a series of publications, Sharp and his colleagues (56,63,65-67), at the University of North Carolina investigated this phenomena and pointed out that the time and concentration of the disinfectant necessary to inactivate the virus will be dictated by the aggregates present. They concluded that aggregates were doubtless a major part of the mechanism for the survival of poliovirus infectivity in treated water (65).

In order to determine the effect of aggregation on poliovirus 1 survival during monochloramine, disinfection tests were done to compare the survival of poliovirus singles and aggregates. Figure 5 is a concentration-time plot showing the relationship of monochloramine concentration and time (in minutes) for 99% inactivation of poliovirus 1 at pH 9 and 15C. This plot is based on individual survival curves with different concentrations of NH_2Cl in the borate buffer system at the same pH (9) and temperature (15C). At pH 9, monochloramine is the predominant species of chlorine present (see Figure 2). The 99% inactivation (i.e., the 1% survival) points used in the construction of the concentration-time plot were obtained from 5 individual survival studies for poliovirus 1 singles and 8 individual survival studies for poliovirus 1 aggregates. Examples of the individual survival curves obtained with poliovirus singles from which 99% inactivation points were obtained are shown in Figure 6 for temperatures of 5, 15, and 25C.

As pointed out by Fair et al. (68), the principal factors that affect the efficiency or rate of destruction or inactivation of a particular species of organisms are: time of contact, concentration of organisms, concentration of disinfectant, temperature, and nature of the disinfectant. The rate of destruction or inactivation of microbes has been usually expressed by a first-order relationship referred to as Chick's Law (69,70),

$$-\frac{dN}{dt} = k_c \cdot N \qquad \text{Eq. 6}$$

where, $-\frac{dN}{dt}$ is the rate of destruction or inactivation expressed as the change in the number of viable microorganisms,

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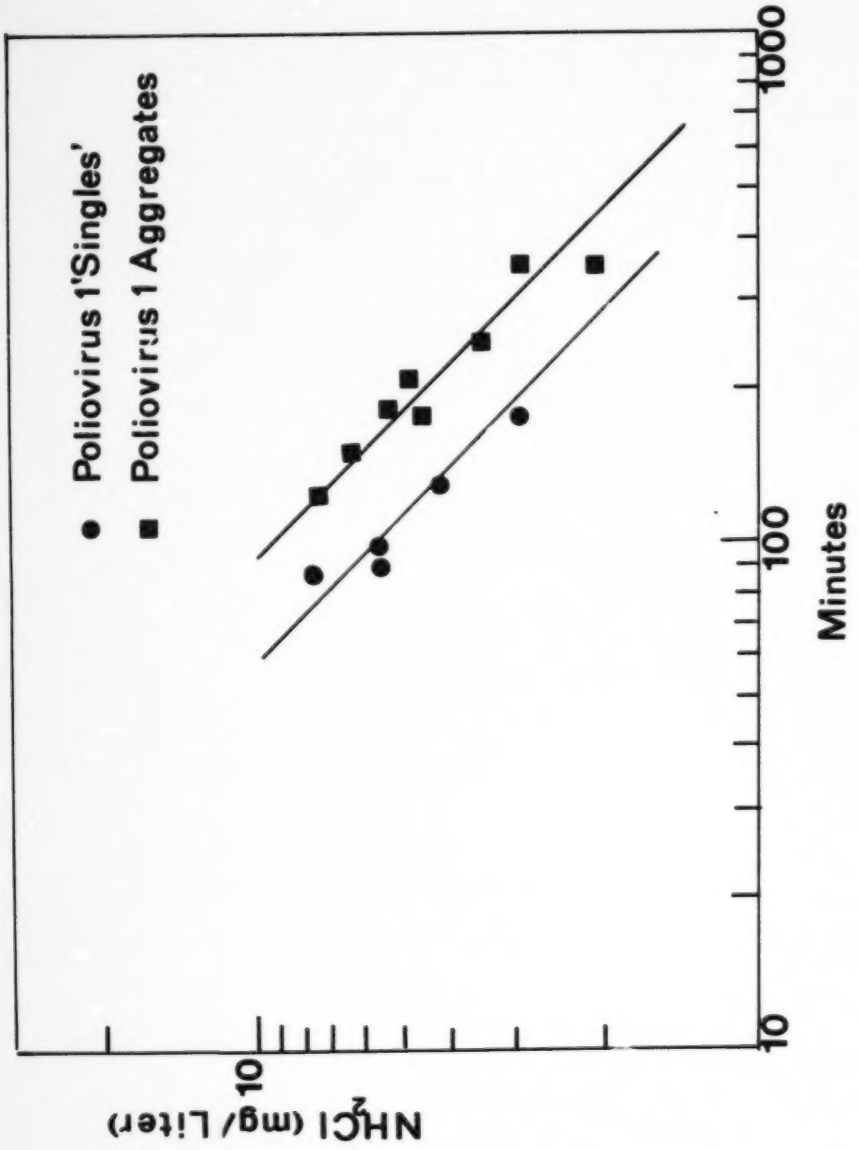

Figure 5. Concentration-time relationship for 99 percent inactivation of poliovirus 1 singles and aggregates by monochloramine at pH 9 and 15C

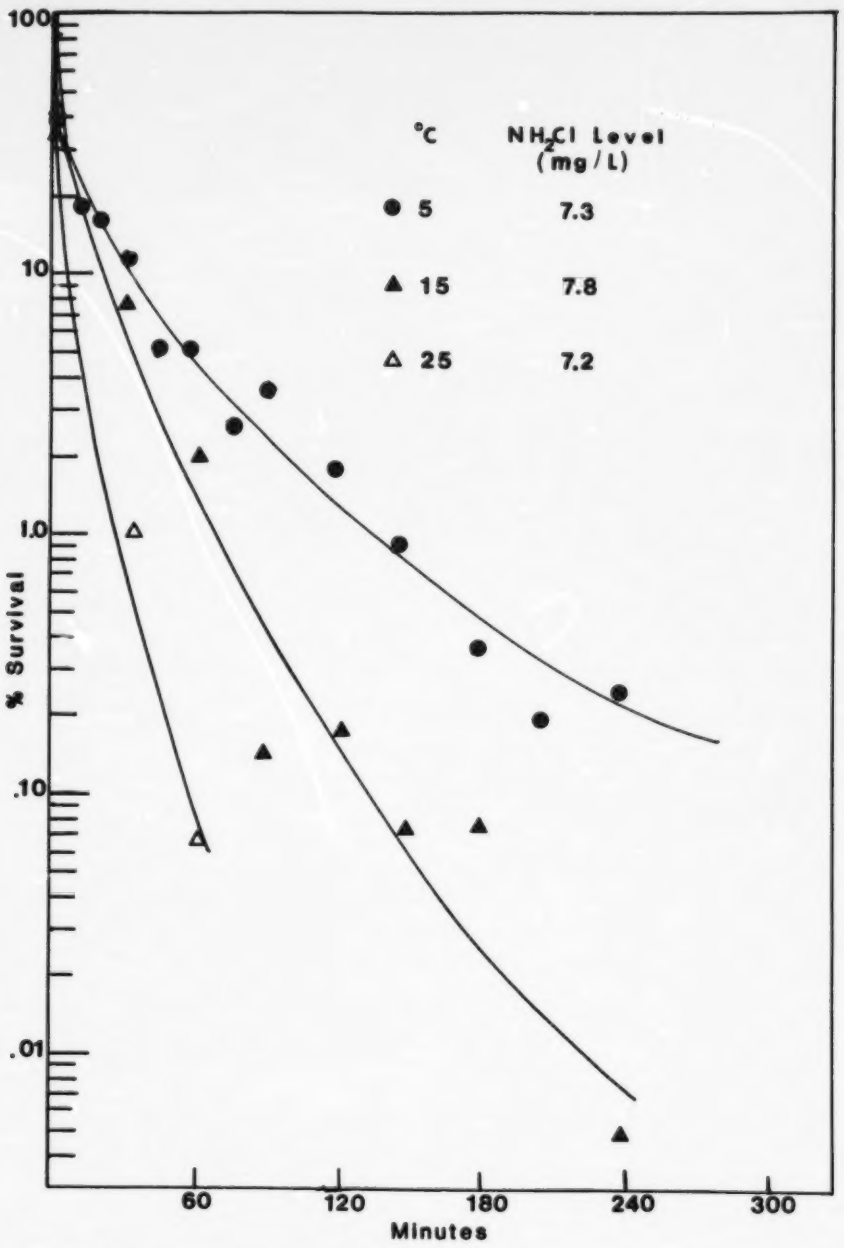

Figure 6. Individual survival curves of poliovirus 1 singles inactivated by monochloramine at pH 9, and at 7.3 mg/L at 5C, 7.8 mg/L at 15C, and 7.2 mg/L at 25C

N, with time t, and k_c is a proportionality rate factor which varies with the disinfectant concentration, temperature, or other conditions, but is independent of the organism number or time.

Integration gives,

$$\log_e \frac{N}{N_o} = - kt \qquad \text{Eq. 7}$$

where, N and N_o are the number of microorganisms living initially and at time, t, respectively; and k is the rate of the reaction, a constant.

Thus, a plot of $\log_e N/N_o$ against t for various contact times should give a linear relationship, i.e., follows first-order kinetics. As illustrated in Figure 6, the logarithm of the percentage of surviving poliovirus 1 singles is plotted as the ordinate, whereas the time in minutes is the abscissa. These studies were done at similar monochloramine levels, but at temperatures of 5, 15, and 25C. Although the process of inactivation was kinetically of the first-order, it should be more properly considered as pseudo-first order (69). As the temperature increased from 5 to 25C at almost the same level of monochloramine, the inactivation rate of the viruses increased, i.e., their inactivation was faster as shown by the time in minutes it took to reduce the poliovirus 1 singles population by 99%. Also, some "tailings" of survivors were observed at temperatures of 5 and 15C.

The concentration-time relationships were plotted on log-log paper, as in Figure 5, using in this case parameters of the level of monochloramine versus the contact time in minutes of the disinfectant. Concentration-time relationships have been expressed in this manner since J.H. van't Hoff (71) demonstrated in 1896 that the disinfectant concentration coefficient, n, indicated the order of the reaction. Chick (69,70) found n to be an exponential function between concentration of the disinfectant and time, i.e.

$$C^n t = \text{constant or } k$$

where, t is the time required to destroy or inactivate a given percentage of organisms (i.e., 99% inactivation of poliovirus 1 singles or aggregates based on individual survival curves), C is the concentration of the disinfectant reactant, n is a constant which is characteristic of a particular disinfectant, and may also be called the concentration exponent, while k is the reaction constant.

Figure 5 shows the plotting of the 99% inactivation points obtained from individual survival curves. The data in all cases formed a straight line that had slopes close to 1, indicating similar first-order inactivation mechanisms. The aggregated poliovirus 1 inoculum was found to be about 1.7

times more resistant to monochloramine than the singles inoculum. This difference was quite significant, since it showed a change in disinfection kinetics of the same virus type due to the viral aggregation.

DICHLORAMINE DISINFECTION OF POLIOVIRUS 1 SINGLES AND AGGREGRATES

A similar difference in disinfection kinetics of the same virus type (i.e. poliovirus 1, Mahoney strain) due to viral aggregration was observed in dichloramine disinfection, when singles viral preparations were compared to aggregated ones. Figure 7 shows the differences in survival characteristics of individual survival curves at similar dichloramine levels (11.22 mg/L for the singles and 12.4 mg/L for the aggregates) at 15C. The singles viral preparation was inactivated at the 90% level approximately eight times as rapidly as the aggregated inoculum. The retardant, stepladder inactivation of the aggregated inoculum in Figure 7 exposed to dichloramine demonstrates the clumped nature of the inoculum.

THE EFFECT OF TEMPERATURE UPON INACTIVATION OF POLIOVIRUS 1 SINGLES BY MONOCHLORAMINE AND DICHLORAMINE

Monochloramine

Since chemical disinfection is a rate process there is an increase in the chemical reaction rate with increasing temperatures. The empirical "rule of thumb" used is that the rate of the reaction increases by a factor of 2 to 3 for each 10-degree rise in temperature. After an extensive review of the literature, Clarke and Chang (41) concluded that the temperature coefficient for a 10-degree change (Q_{10}) in the destruction of virus by free chlorine increased the rate of virus inactivation by a factor of 2 to 3 (200 to 300 times). In these present studies, Figure 8 shows the concentration-time relationship for 99% inactivation of poliovirus 1 singles at temperatures of 5, 15, and 25C by monochloramine at pH 9. Although all three curves illustrated linear reaction rates, they were not equally spaced. A 10-degree temperature change from 5 to 15C showed a Q_{10} of 1.5, whereas that from 15 to 25C was 4, giving an average Q_{10} value of 2.75. Poliovirus 1 singles were inactivated faster at the higher temperature of 25C when 99% inactivation points were used to construct the concentration-time figure. However, when the NH_2Cl data were regraphed using 90% inactivation points (see Figure 9), all three curves were equally spaced. The Q_{10} from a temperature of 5 to 15C was now 2, while that from 15 to 25C was 1.9, giving an average Q_{10} value of 1.95. The lower parts (the "tails") of the monochloramine survival curves at temperatures especially of 5 and 15C (see Figure 6) were in part non-linear on semilogarithmic plots, whereas the upper portions (the 90% inactivation points) reflected first-order kinetics. Although poliovirus 1 singles were used in these studies, the observed "tailings" were similar to those seen with aggregated preparations of poliovirus. The non-linearity (i.e., "tailings") of the survival curves appeared more frequently at lower temperatures (i.e., 5 and 15C) rather than at the higher temperature of 25C.

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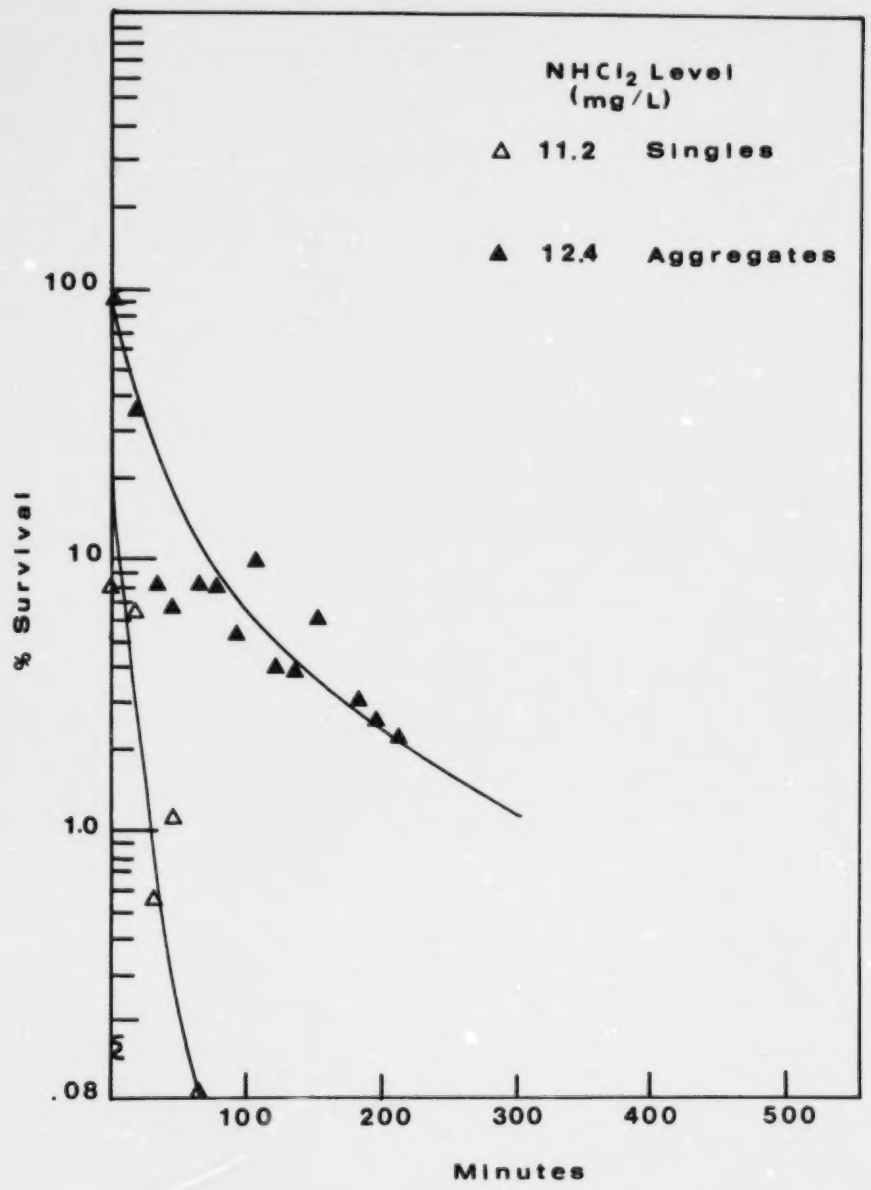

Figure 7. Individual survival curves comparing poliovirus 1 singles versus aggregated inocula at similar dichloramine levels (11.2 mg/L for the singles and 12.4 mg/L for the aggregates) at 15C and pH 4.5

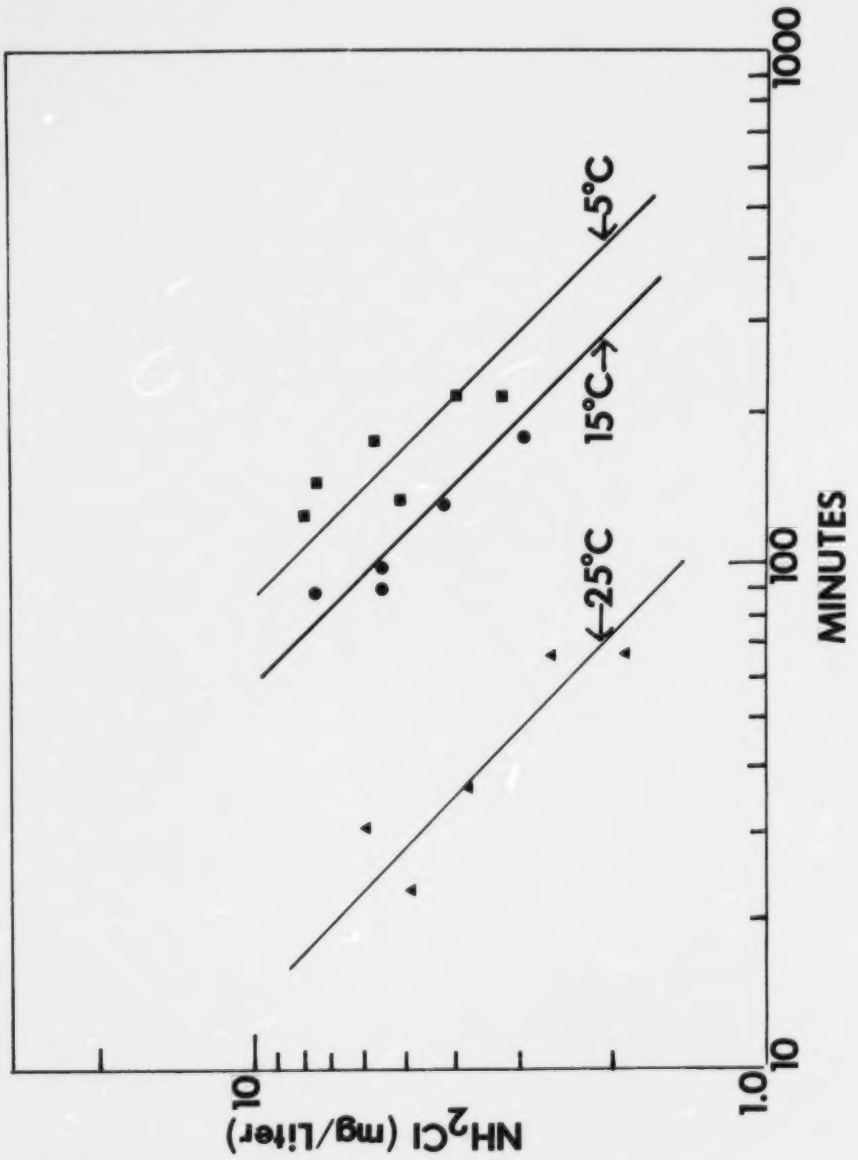

Figure 8. Concentration-time relationship for 99% inactivation of poliovirus 1 singles by monochloramine at pH 9 at temperatures of 5, 15, and 25C

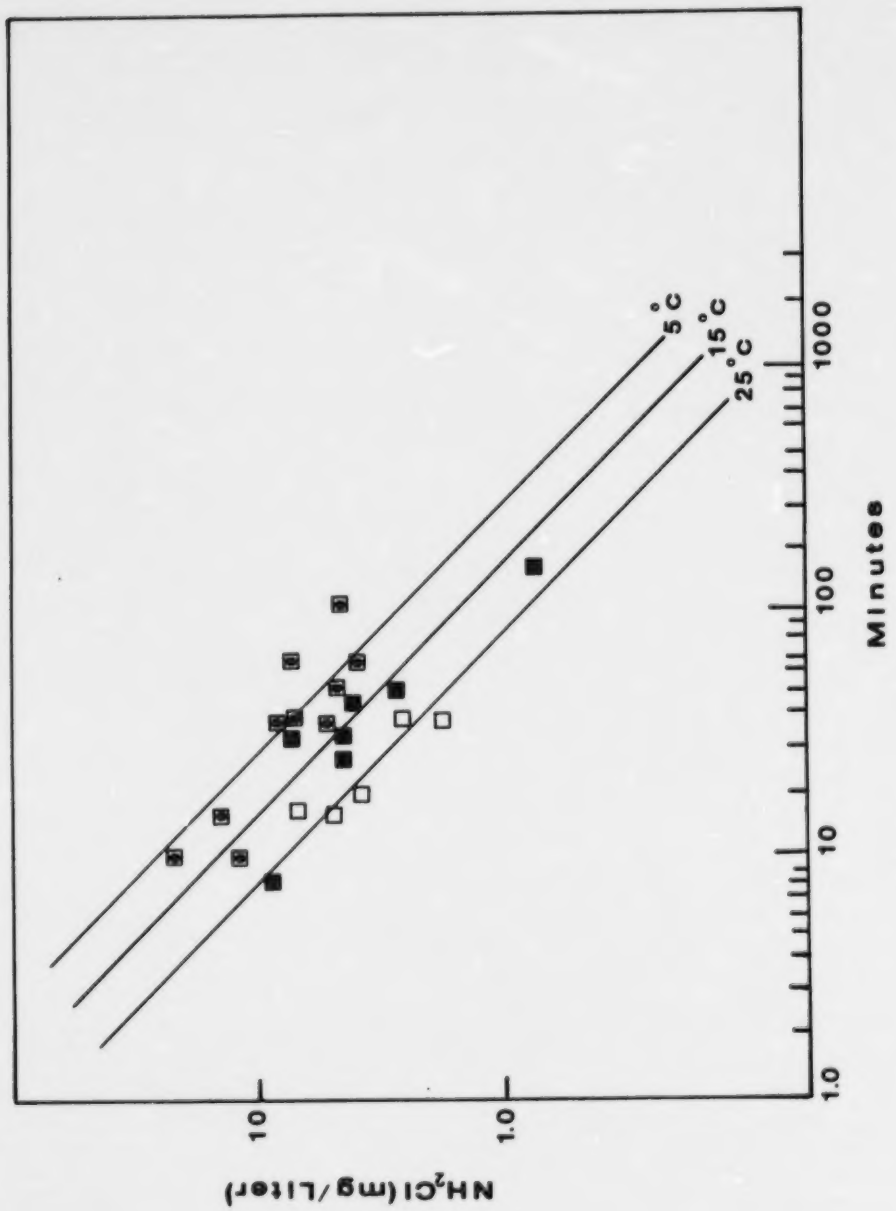

Figure 9. Concentration-time relationship for 90% inactivation of poliovirus 1 singles by monochloramine at 5, 15, and 25C at pH 9

Dichloramine

Individual dichloramine ($NHCl_2$) survival curves of poliovirus 1 singles at both 5 and 15C showed more extended kinetic rate patterns than those encountered with monochloramine (Figure 10). The rate of inactivation initially followed first-order kinetics for at least part of the survival curve. In several cases there was evidence of a retardant rate process, where the survival curve demonstrated "tailing" of the survivor fraction of the original inoculum. This might have been due to the presence of poliovirus clumps or aggregates still in the inoculum. Clumping would restrict the penetration of the disinfectant into the aggregates and thus enable internal viral survival to occur in the tissue culture recovery system.

Although so-called "singles" preparation of poliovirus 1 were used in this work, clumps of virions were still evident. The freon extracted-density gradient method of preparing poliovirus singles was found in a previous study (65) to yield no detectable cellular debris upon examination by electron microscopy, and to be composed of 93.1% single virus particles and 6.9% aggregated virions. The aggregated viral fraction was further characterized and found to have 3.9% of the virions in pairs and 3.0% existing in a state of $\pm$ 5 virus particles. The aggregated poliovirus inoculum contained, on the other hand, considerable cellular debris, a virus content of 90.7% single virions, and 9.3% aggregated forms. The aggregated virus fraction consisted of 4.4% pairs, 0.1% triplets, and 4.8% of $\pm$ 5 virus particles. Thus, the difference (except for the cellular debris) was not great; but was significant when aggregates and singles inocula were compared (Figure 5). The dichloramine (and monochloramine) survival curves were similar in shape; as the percentage of recovered virus particles declined, there occurred a gradual slowing of the inactivation rate followed by a leveling off of the curve. Survival curves of this type are usually indicative of interference in the die-away kinetics of the process (Chick's Law) and were due to the presence of clumps or aggregates of virions in the inoculum. Although the fractional rate of inactivation of the virus for a given set of conditions should be a constant according to Chick's Law, deviations from Chick's Law occur, as documented here with aberrant survival curves. Chang's (72,73) multi-Poisson distribution model for treating disinfection data illustrates the inactivation of increasing virus clump sizes; i.e., the smallest clump size or single virions were inactivated first since more surface area per virion was exposed to the disinfectant, followed by the inactivation of increasing clump sizes. Thus, the inactivation rate appeared to slow down as larger and larger clumps were encountered until the survival curve "tailed off".

Figure 11 is a log-log plot of the concentration-time relationship for 90% inactivation by dichloramine of poliovirus 1 singles at 5 and 15C. A 10-degree increase in temperature gave a Q_{10} of 2.5 for poliovirus 1, which was within the 2 to 3 factor increase noted by Clarke and Chang (41). When the data from Figures 9 and 11 were combined in Figure 12, the dichloramine 90% concentration-time relationships did not parallel those encountered for monochloramine. The inactivation kinetics may thus be different.

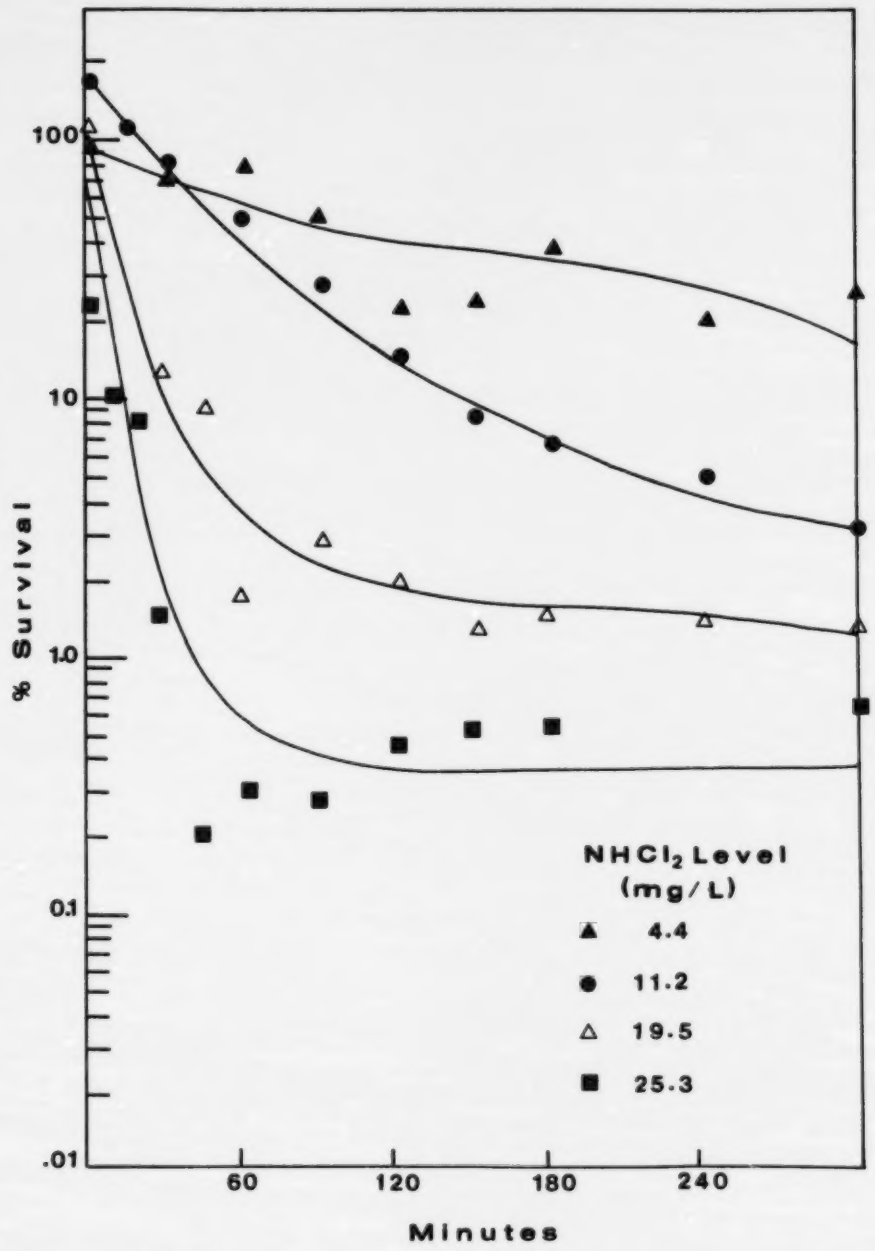

Figure 10. Individual survival curves of poliovirus 1 singles inactivated by dichloramine at pH 4.5 and 5C, and at concentrations of 4.4, 11.2, 19.5, and 25.3 mg/L

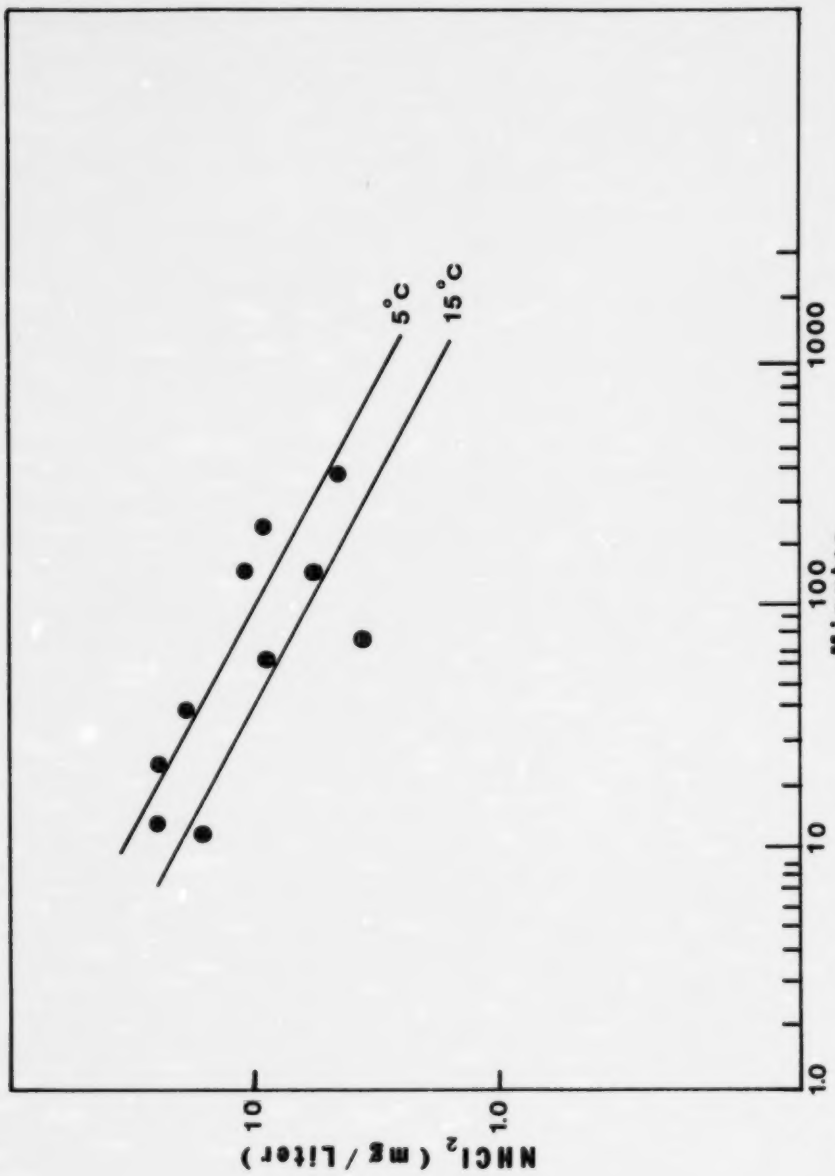

Figure 11. Concentration-time relationship for 90% inactivation of poliovirus 1 singles at temperatures of 5 and 15C by dichloramine at pH 4.5

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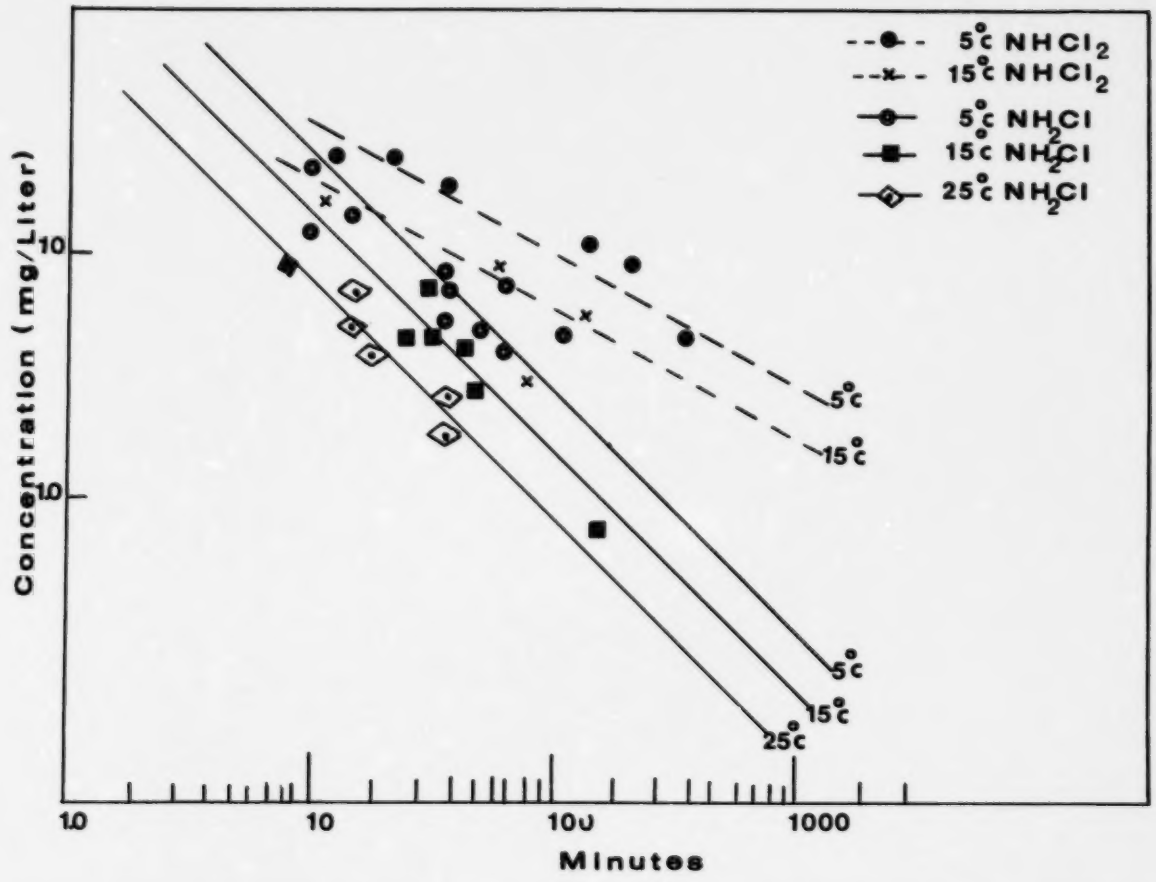

Figure 12. Comparison of the concentration-time relationships for 90% inactivation of poliovirus 1 singles by monochloramine at 5, 15, and 25C at pH 9, and by dichloramine at 5 and 15C at pH 4.5

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THE EFFECT OF pH UPON THE INACTIVATION OF POLIOVIRUS 1 SINGLES AND ESCHERICHIA COLI BY MONOCHLORAMINE

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The pH range of most mineral-bearing waters are generally within the narrow range of 6 to 9 (74). The Classes AA and A used by the State of New York for fresh surface waters have a recommended pH range between 6.5 and 8.5, and after appropriate treatment are considered safe and satisfactory for drinking water purposes (75). Most finished drinking waters in the United States are maintained at a pH level below 9, usually between 7 and 9 (76). At pH values of 9 and above, the chloramine that is formed when hypochlorous acid reacts with ammonia is predominately monochloramine. Thus, many research studies are done at pH 9 or above. However, pH values lower than 9 can be encountered in drinking water treatment. In order to evaluate whether a still predominately monochloramine system at a pH lower than 9 is a better disinfectant, the following procedure was followed.

Levels of monochloramine were first preformed at pH 9 in 0.05 M borate buffer. The pH of this preformed monochloramine was then dropped immediately to 7 with 0.2 N HCl. The monochloramine was found to be stable at this pH for greater than four hours (see Table 3). This confirms previous studies by Snead and Olivieri (77). If the chloramines had been initially formed at pH 7, the chloramines present would have been a mixture of both monochloramine and dichloramine. By forming the chloramines at pH 9, and then dropping the pH to 7, it was now possible to determine the effect of a lower pH along with mostly monochloramine upon the inactivation of poliovirus 1. As a comparison, the study was also done using the test bacterium Escherichia coli. The elucidation of the effect of solely a pH change to 7 was now possible. Figure 13 therefore compares the disinfection of poliovirus 1 singles at 5C by 11 mg/L of preformed monochloramine adjusted to pH 7 compared to 10.8 mg/L of monochloramine formed and held at pH 9. The change in pH had no apparent effect on the disinfection of the poliovirus 1 singles. This study was repeated using E. coli as the test organism. In this study (shown in Figure 14), monochloramine levels of 2.0 mg/L at pH 7, and 2.2 mg/L at pH 9 were tested. Contrary to what occurred with poliovirus, the lowering of the pH from 9 to 7 increased the rate of monochloramine disinfection efficiency (at the 99% inactivation level) about ten times. Figure 15 is another comparison study of the disinfection at 5C of poliovirus 1 singles with monochloramine at pH 9 and 7. The E. coli results shown here are from Figure 14. Again, as seen in Figure 13, there was a distinctly different pattern of inactivation for the virus than for the bacterium. At similar concentrations of monochloramine the virus was inactivated at the same rate. On the other hand, approximately the same concentration of monochloramine inactivated the test bacterium more rapidly at pH 7 than pH 9. Thus, monochloramine was shown to be a better disinfectant for bacteria at pH 7 than at 9, but this was not so in the case of poliovirus 1.

Viruses are generally considered to be stable in the pH range of 5 to 9 encountered in natural waters, whereas the growth of bacteria is usually characterized by a similar range but have a pH optima near neutrality. The effect of pH upon transport mechanisms across the bacterial cell membrane may have influenced the greater monochloramine destructive effect at pH 7 compared to pH 9. For example, Chang (78,79), while studying the inactiva-

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TABLE 3. Stability of Monochloramine at pH 7

Time after formation[a] (hr)	NH_2Cl content (mg/L)
0	11
1.0	11
2.5	11
3.0	11
3.5	11
4.0	11

[a]The pH of preformed NH_2Cl was dropped to pH 7.0 immediately after NH_2Cl formation.

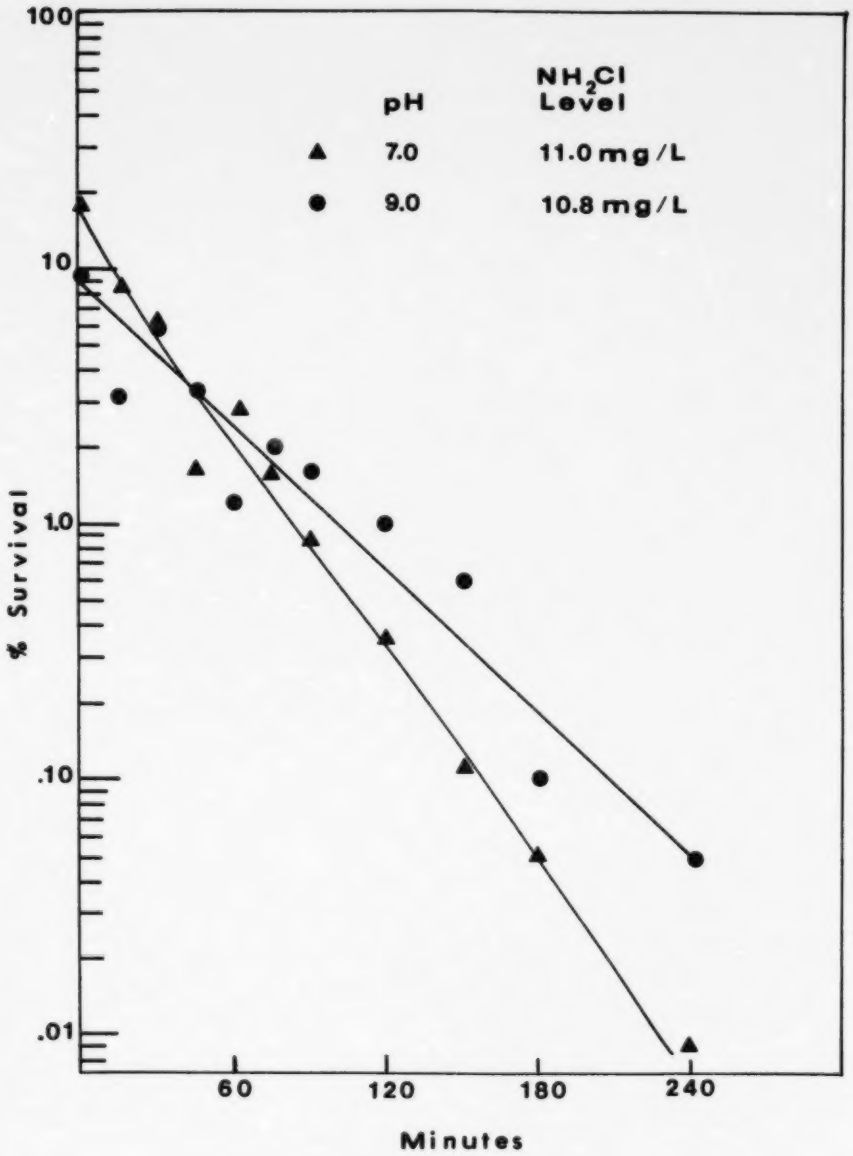

Figure 13. Inactivation of poliovirus 1 singles by monochloramine at 5C at pH 9 and pH 7 (preformed at pH 9) by similar concentrations of the disinfectant

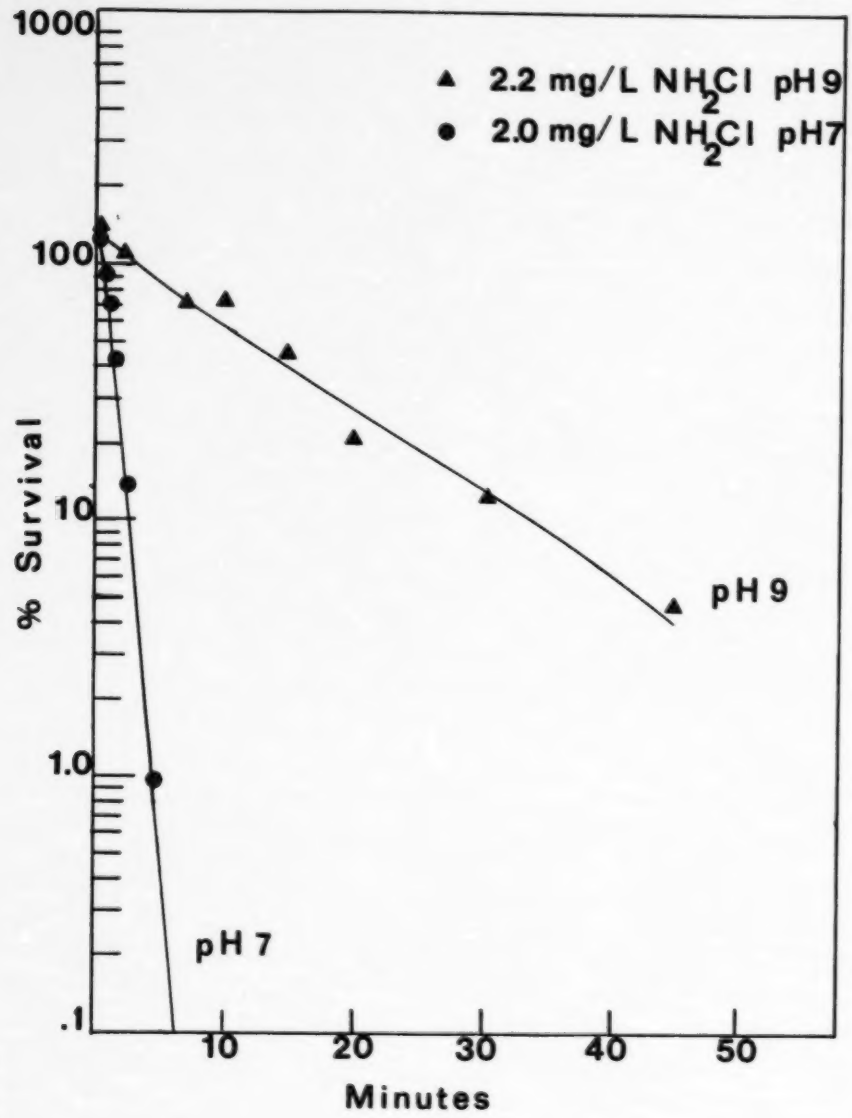

Figure 14. Disinfection of Escherichia coli by monochloramine at 5C at pH 9 and 7 (preformed at pH 9) by similar concentrations of the disinfectant

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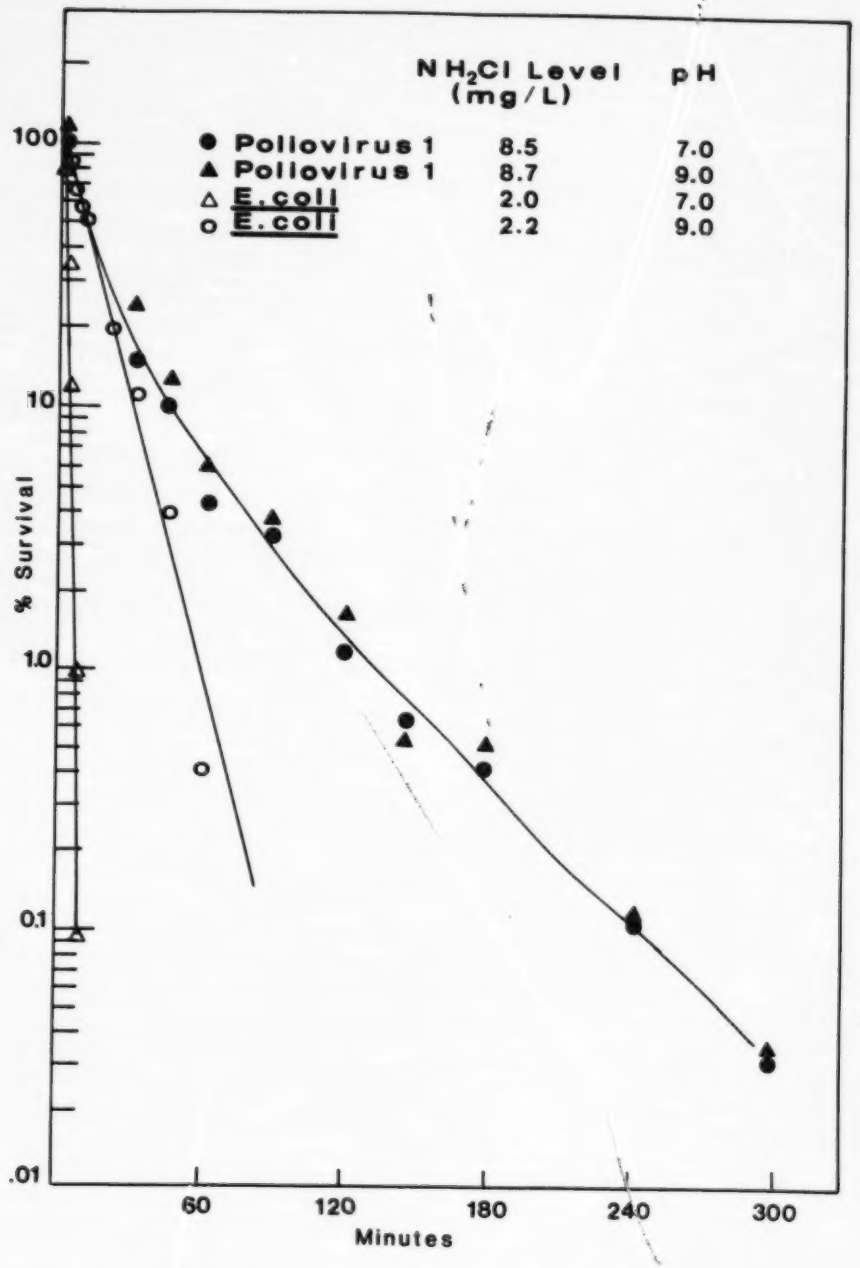

Figure 15. The inactivation of poliovirus 1 singles and Escherichia coli at 5C by monochloramine at pH 9 and pH 7 (preformed at pH 9)

tion of Entamoeba histolytica cysts by chlorine, noted greater uptake of chlorine and less survival at low pH than at high pH. Dennis (80), in a more recent study, found that the inactivation rate of the bacterial virus f2 increased with decreasing pH. The incorporation of chlorine into the f2 bacterial virus was dependent on pH, and the higher rates of incorporation occurred at lower pH values. The fact that in my study poliovirus 1, an animal virus, did not demonstrate a similar effect as that noted by Dennis with the bacterial virus points out once again the caution that should be displayed when using a surrogate animal virus, such as the f2 bacteriophage. These studies should be continued using other animal viruses, to determine if there is greater inactivation by monochloramine at pH 7 compared to pH 9.

COMPARISON OF THE DISINFECTION OF ESCHERICHIA COLI USING PREFORMED AND FORMING MONOCHLORAMINES

Chloramine research studies usually utilize preformed monochloramines as the disinfectant. The combination of ammonia (NH_3) with chlorine (Cl_2) to form chloramines for the treatment of drinking water was practiced for many years. Ammonia is still deliberately added to some chlorinated public water supplies to provide a combined available chlorine residual (i.e. chloramines). Monochloramine is the principal chloramine that is encountered in drinking water treatment. Monochloramine formation is very rapid at the concentrations and conditions of water treatment, the reaction (shown in Eq. 3) being usually 90% complete in about 1 minute (81,82). As shown in Figure 2, the reaction rate is maximum at the pH range 8.5 to 9.0 (81,82). Chloramines are less effective oxidizing and disinfecting agents than free chlorine residuals (i.e., hypochlorous acid and hypochlorite ion), but they are more stable and can retain a residual in water for a longer period of time. Chloramines can, however, be successfully used, and even preferred, under certain use conditions. Since many waste waters contain large amounts of nitrogenous substances, chloramines are usually the only form of chlorine present unless breakpoint chlorination is practiced. In recent years chloramines have not been recommended as a primary disinfectant because of low germicidal efficiency. Criticism has been directed towards this decision of the U.S. Environmental Protection Agency (83). It was pointed out that under field conditions, disinfectants with high lethality coefficients may not be in the final analysis as good as those, such as chloramines, with lower lethality coefficients (83). Thus, factors such as raw water quality, the presence of oxidizable compounds, the contact times, the construction of the chlorine contact chamber to maximize chlorine feed solution mixing rates, and the ammonia to chlorine application ratios (for chloramines) must all be evaluated and specific guidelines developed to maximize disinfection efficiency (83,84).

Another concern that has been expressed by some is that forming monochloramines were better disinfectants than the application of preformed monochloramines. Thus, this study attempted to cast more light upon the "real world" situation where monochloramines are formed during the process of disinfection and are not added in the preformed state. The disinfecting efficiencies of preformed monochloramine (NH_2Cl) and forming monochloramine (free chlorine and NH_3) against the test bacterium E. coli were compared in the same study, along with reference to the disinfecting ability of free

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chlorine alone. In the forming monochloramine study, the pH 9 buffer was first dosed with sufficient free chlorine to give a 1.86 mg/L concentration, then sufficient ammonium sulfate was added to produce an ammonia to chlorine ratio of 6:1 (by weight). The ammonia solution and the E. coli inoculum were added at the same time. A previous study showed that the E. coli was not adversely affected by the ammonia alone. The results of these studies are shown in Figure 16. The tests were done at 5C and pH 9. The forming monochloramine was found to be more effective (about 1.2 times as effective) than the preformed monochloramine. Split-second exposure of the E. coli inoculum to the hypochlorite-hypochlorous acid mixture which existed at pH 9 in the forming monochloramine study may have been responsible for the initial faster kill of the test bacteria. This conclusion is made because when a mixture of hypochlorite ion and hypochlorous acid at pH 9 (OCl^- + HOCl curve in Figure 16) was used as a disinfectant in a separate test in this study, the latter disinfectants destroyed 99% of the inoculum within a contact time of 3 minutes. In the first 3 minutes in the forming monochloramine curve in Figure 16, 30% of the original bacterial inoculum was destroyed, leaving fewer bacteria to be disinfected by the forming monochloramines. The preformed monochloramine curve showed a "hump" in the initial part of the curve, but then after 15 minutes the bacteria were destroyed in a linear first order fashion. No "hump" was observed in the forming monochloramine study, and after 15 minutes this latter curve paralleled the preformed monochloramine destruction curve. These results showed that the disinfection rates for the forming and preformed monochloramines were the same after the first 15 minutes of the study; the observed differences in the positioning of the lines can be attributable to the differences in bacterial numbers after the first 3 minutes of the study. Although the original bacterial inocula were similar, there was a greater initial kill of the bacteria in the forming monochloramine study than in the case of the preformed monochloramine. After the first 3 minutes of the forming monochloramine experiment, the ability of the newly formed monochloramine to kill the remaining bacteria was the same as that encountered in the preformed study, but there were more bacteria to disinfect in the preformed study. It appears that the brief initial exposure of the bacteria in the inoculum to the free chlorine present before the monochloramine was completely formed accounts for the differences between the two monochloramine survival curves.

SEQUENTIAL ADDITION OF POLIOVIRUS 1 TO DETERMINE THE EXTENT OF MONOCHLORAMINE DISINFECTING EFFICIENCY

Survival curves seen in these studies often show retardant die-away/inactivation patterns. Although the disinfectant level was never depleted during the course of the experimentation, the question arose whether changes had nevertheless occurred in the disinfectant's efficiency which would account for retardant curves. Thus, a second inoculum of poliovirus 1 was added two hours after the first virus administration (see Figure 17) to determine if the inactivation rate of this subsequent virus inoculum would mimic the first portion of the curve. As seen in Figure 17, there was a reappearance of the rapid initial inactivation rate, indicating that the disinfecting efficiency of the original monochloramine present had not been effected or altered, and that this monochloramine was still capable of inactivating the additional inoculum.

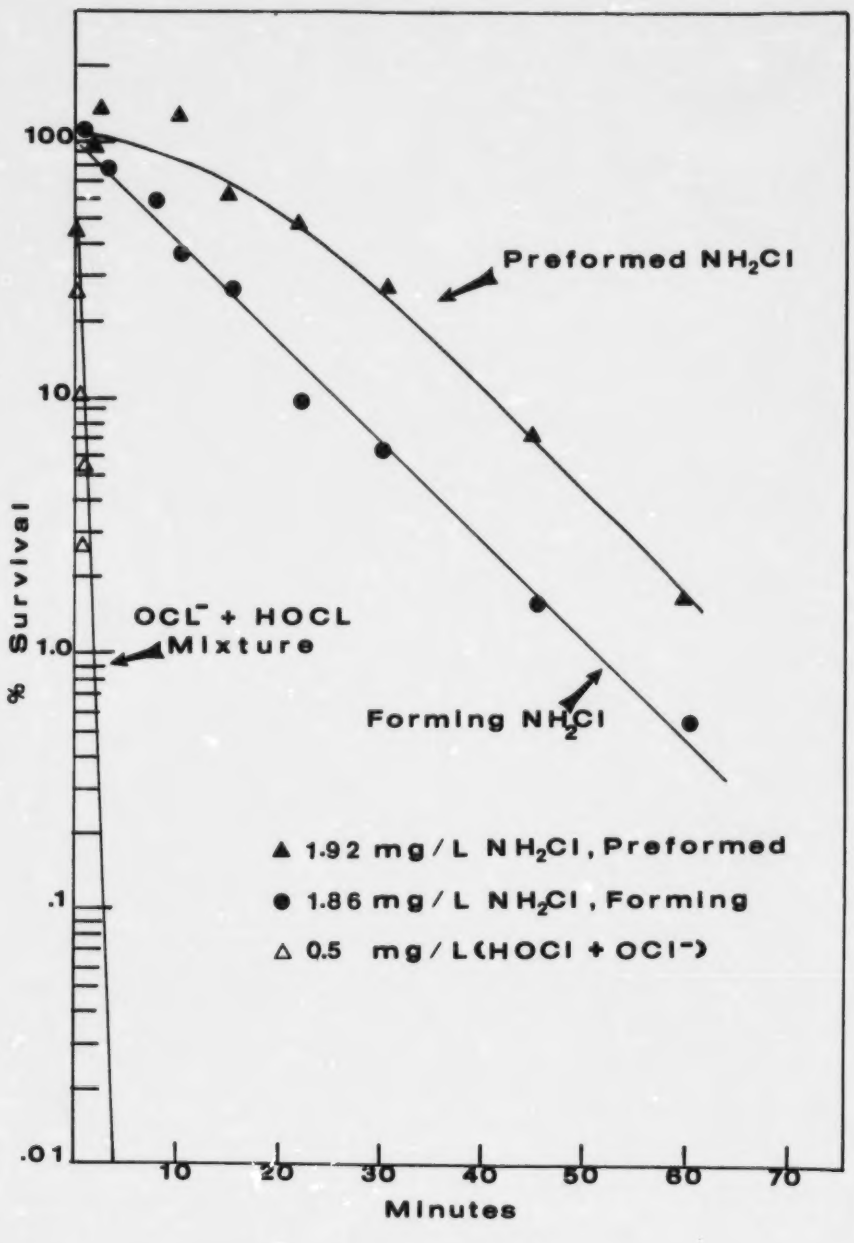

Figure 16. Disinfection of Escherichia coli 11229 at 5C and pH 9 by forming and preformed monochloramines compared to a 0.5 mg/L mixture of hypochlorous acid (HOCl) and hypochlorite ion (OCl^-)

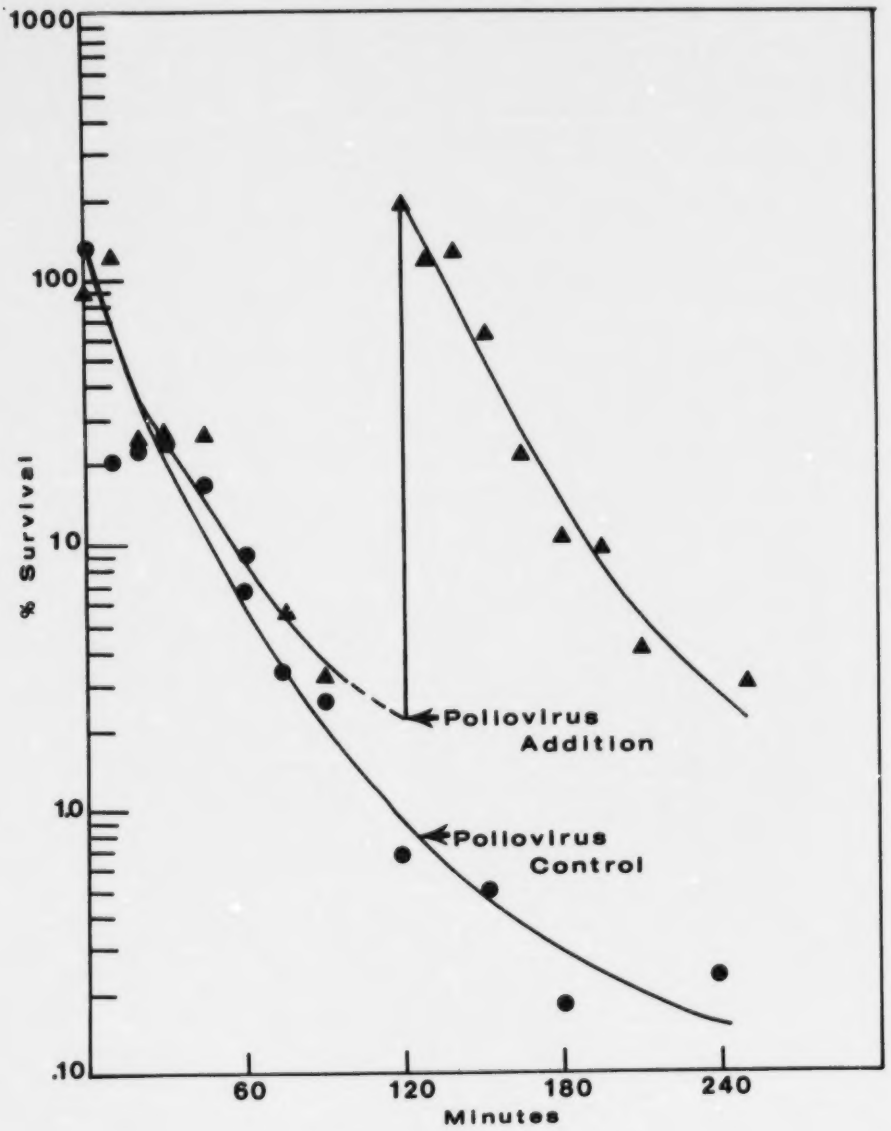

Figure 17. Disinfecting efficiency of 10.8 mg/L monochloramine at 5C and pH 9 to inactivate a sequential addition of poliovirus 1 singles

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THE EFFECT OF INCREASING CONCENTRATIONS OF MONOCHLORAMINES UPON THE INACTIVATION OF POLIOVIRUS 1 SINGLES

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In the process of this research, it was noted that increasing the monochloramine concentration did not proportionally increase the rate of inactivation of poliovirus 1 singles. Figure 18 shows the effect of the almost doubling of the monochloramine level from 12 to 22 mg/L at 5C and pH 9. Although 12 mg/L of monochloramine was about 4 times more effective at the 99% inactivation point than the lower concentration of 5.4 mg/L monochloramine, 22 mg/L was found to be as effective as 12 mg/L. This was contrary to the work of Butterfield and Wattie (85) who believed that "without exception" an increase in the amount of chloramine present increased the rate of kill of their test organism, E. coli. However, what they termed "marked" increases in the extent of kill was not observed in 60 minutes of exposure at 2 to 6C, with less than 1.2 mg/L residual at pH 8.5, and 1.5 mg/L residual at pH 9.5. At the higher temperature range of 20 to 25C that was used they found that monochloramine residuals of about 0.3 and 0.6 mg/L were required to obtain about the same kill rate at pH 8.5 and 9.5, respectively. On the other hand, Snead and Olivieri (77), while studying the inactivation of f2 bacterial viruses by monochloramine at 25C and pH 7, found that above a monochloramine concentration of 4.0 mg/L the extent of f2 inactivation was relatively independent of the monochloramine concentration. Below 4.0 mg/L the degree of inactivation was found to be dependent on the monochloramine concentration. My work described in this report is more consistent with the results of Snead and Olivieri (77) than Butterfield and Wattie (85), but differences might be due to the use of viruses versus bacteria as the test organisms.

THE EFFECT OF CHLORIDE IONS UPON MONOCHLORAMINE DISINFECTION OF POLIOVIRUS 1 SINGLES AND ESCHERICHIA COLI

Studies by Scarpino et al. (1), and confirmed by Engelbrecht et al. (86) and Jensen et al. (87), found that poliovirus 1 (Mahoney) was inactivated more rapidly by chlorine in the form of hypochlorite ion (OCl^-) at pH 10 than by hypochlorous acid (HOCl) at pH 6. Scarpino et al. (1) suggested that the borate buffer system (containing KCl) had an influence on the hypochlorite ion and hypochlorous acid virucidal relationships. Since 0.2 N HCl had been used to prepare preformed monochloramie at pH 9 (see The Effect of pH upon Inactivation .... in Section 5)), it was decided to investigate the effect of the chloride ion on the disinfection process. The addition of 0.2 N HCl made the 0.05 M borate buffer system about 0.02 M with respect to chloride ions. A study was thus done testing the presence and absence of 0.02 M chloride ions, added as the sodium salt (0.02 M NaCl), on the inactivation by monochloramine of poliovirus 1 singles at 5C and pH 9 using borate buffer prepared without KCl. The results in Figure 19 (characteristic not only of this study but one other) showed that the chloride ions had little or no effect on the monochloramine disinfecting process, since the survival curves closely paralleled each other. If the chloride ions (as 0.02 M NaCl) had influenced disinfection, a difference in inactivation kinetics would have been apparent. A similar study with E. coli was also performed at 5C (see Figure 20) where 3.2 mg/L monochloramine was formed at pH 7, 0.02 M chloride ions were added as the sodium salt, and disinfecting comparison was made to the same level of

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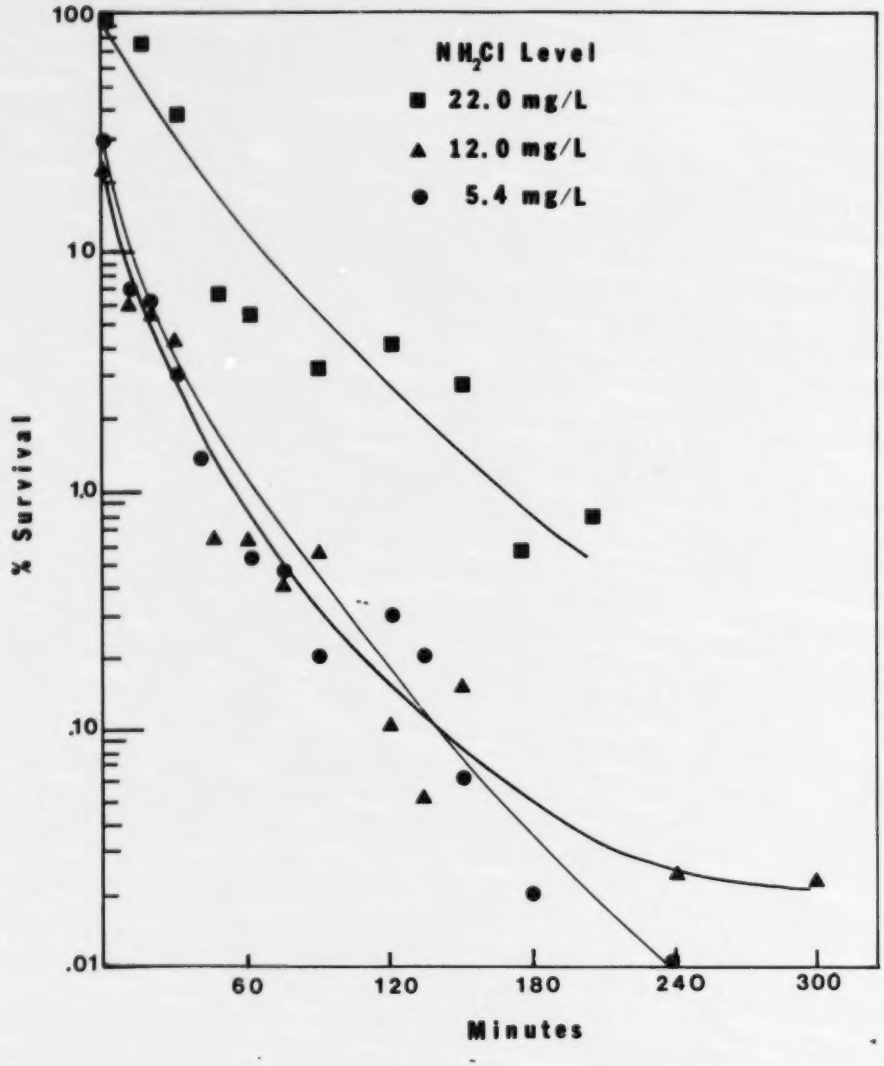

Figure 18. The inactivation kinetics of poliovirus 1 singles with increasing monochloramine at 5C and pH 9

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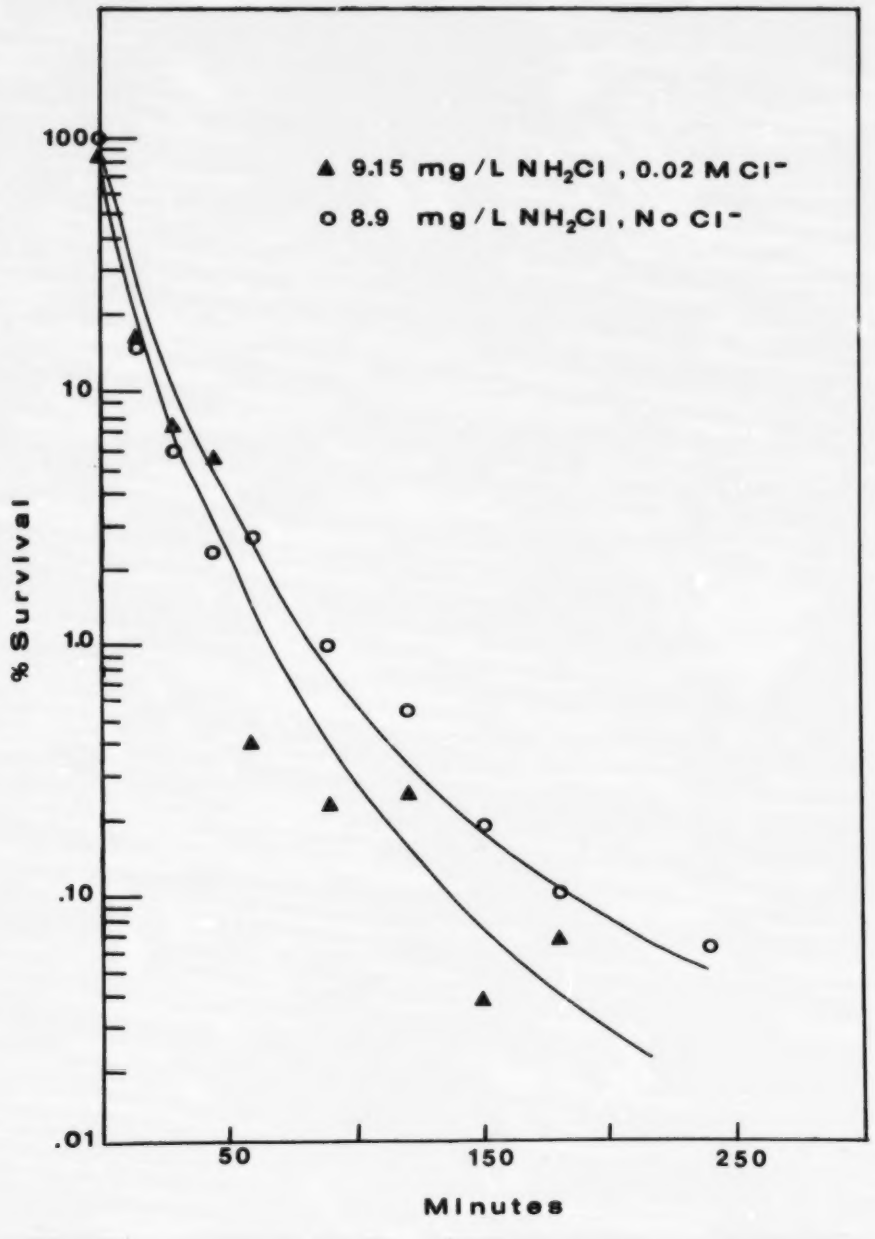

Figure 19. Inactivation of poliovirus 1 singles at 5C and pH 9 by monochloramine with and without the addition of 0.02 M chloride ions as the sodium salt

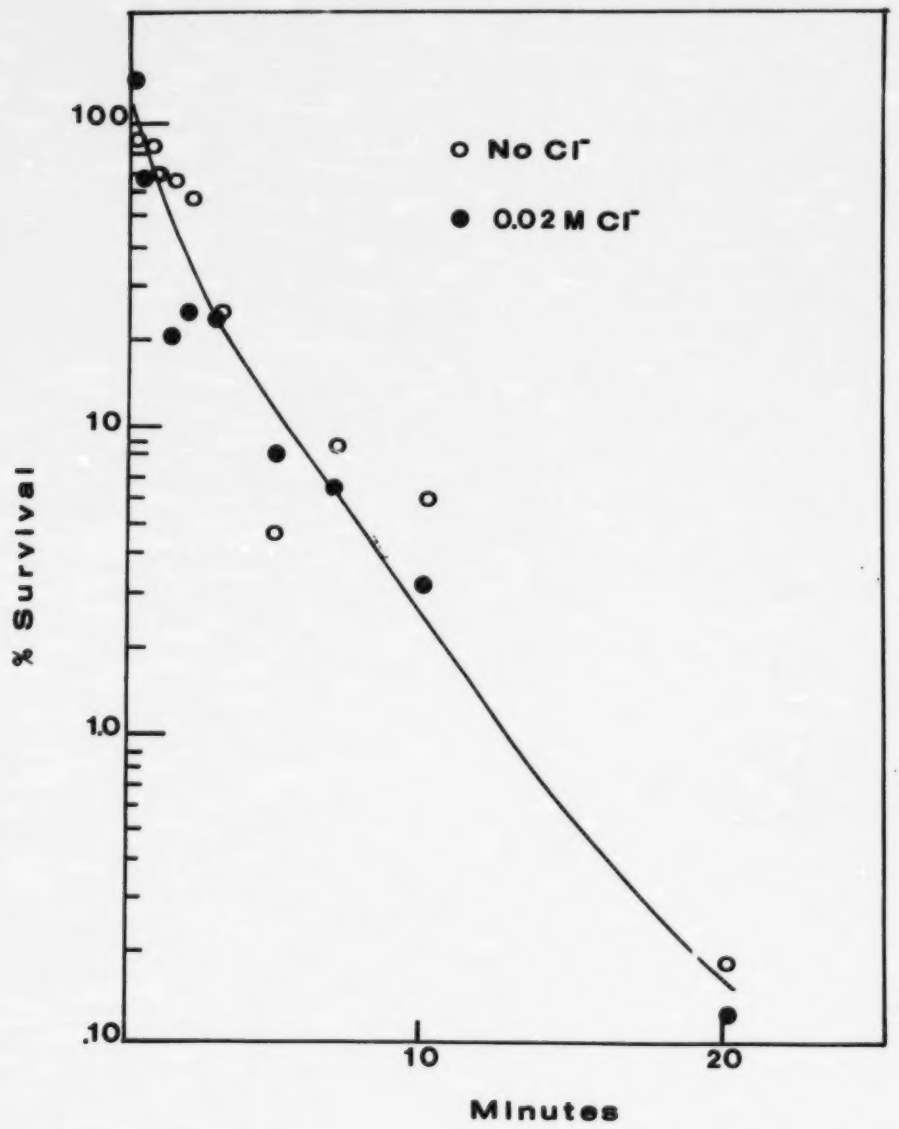

Figure 20. Disinfection of Escherichia coli 11229 at 5C and pH 7 by 3.2 mg/L monochloramine with and without the addition of 0.02 M chloride ions as the sodium salt

monochloramine at pH 7 but without the added chloride ions. No effect of the added chloride ions was observed. Therefore, the observed (Figure 14) difference in disinfection at pH 9 and pH 7 for E. coli was due to the pH change to 7 and not due to the addition of chloride ions when the 0.2 N HCl was added to the buffer system.

SELECTION FOR MONOCHLORAMINE-RESISTANT POLIOVIRUS 1

Bates et al. (22) reported that a laboratory strain of poliovirus 1 (LSc) could be made resistant to free chlorine at pH 7 after repeated exposures to initial free chlorine levels of 0.8 mg/L for up to 30 minutes of inactivation. They exposed the virions to free chlorine, grew the survivors, and then reexposed them to the free chlorine. After ten such exposures, their data suggested increased resistance to free chlorine. There was a gradual enhancement of resistance to the free chlorine doses over several cycles of exposure to free chlorine, rather than a single-step process of resistance. They believed that this gradual development of resistance suggested an evolutionary or adaptive alteration in the virus population after repeated sublethal exposures to free chlorine. Subsequently, Bates et al. (88) exposed the same poliovirus strain previously used to 10 cycles of inactivation by chlorine at pH 5 and pH 9 and compared their results to the data from their earlier research (22). Virus exposed to chlorine at pH 9 (as mostly hypochlorite ion) demonstrated more progressive development of resistance than to the chlorine at pH 5 (as mostly hypochlorous acid). They reported that polioviruses with resistance developed at pH 7 showed rates of inactivation when exposed to chlorine at pH 9 similar to those of the virus which was repetitively exposed to chlorine at pH 9. This suggested to them that the mechanism of resistance development was the same at pH 7 and pH 9. The development of poliovirus resistance at pH 5 to chlorine was not as apparent as at pH 7 and 9. This was explained by them on the basis of the faster inactivation rate in all the cycles at pH 5, which was due to the predominate chlorine species, hypochlorous acid. They concluded that increased amounts of hypochlorous acid or environmental conditions on the acid side of neutrality would not favor selection of resistant viruses but would favor virus inactivation. Also, at pH 7 and 9 where hypochlorite ion dominated as the chlorine species, virus inactivation occurred more slowly and facilitated the detection of virus plaques containing the most resistant virus survivors. If the latter conclusion is correct, monochloramine (a slower combined chlorine disinfectant than hypochlorite ion) could also be successfully used as the chlorine species at pH 9 to develop resistant poliovirus. However, increased resistance reported by Bates et al. (22) might have been partly due to the formation of aggregates in the virus suspension. In an attempt to prepare a monochloramine resistant poliovirus we guarded against the possible effect of aggregates influencing the appearance of "resistant" variants by using the procedure of Floyd et al. (56) for poliovirus singles preparation as well as the Bates et al. (22) procedure. Both procedures used for preparation of the virus inocula are outlined in Figure 3, page 19, of this report. The poliovirus prepared by both procedures were exposed separately under the same test conditions for similar time periods. After exposure to monochloramine, the more resistant plaques were isolated and regrown and then re-exposed to monochloramine. Eight repetitive mono-

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chloramine exposure cycles were performed for viruses prepared by both procedures.

Tables 4 and 5 summarized the results of these studies. The studies of Table 4 with the Bates et al.-prepared viruses (22) did not show the development of resistance to monochloramine. For instance, Figure 21 shows the plotted % survival data of study 10 (in Table 4) where viruses exposed 7 and 8 times to monochloramine were combined as the inoculum. The latter had to be combined because of the low numbers grown-out of poliovirus survivors in study 9. Comparison was made in this Figure to that obtained with the Bates et al.-prepared virus (22) which had never been exposed to monochloramine. No differences between the survival curves were apparent. However, the Table 5 survivor data showed development of virus resistance using the Floyd et al.-prepared virus (56) exposed to monochloramine. Although the viral resistance patterns were at time irregular, such fluctuations might be due to the possible heterogeneity of resistance of virus in separate surviving plaques, as noted by Bates et al. (22). Using this base of reasoning, this would explain the demonstration of resistance to monochloramine as plotted in Figure 22 from the percent survival data in studies 8 and 10. The virus in study 8 had been exposed 7 times to monochloramine, whereas that in study 10 was exposed 8 times. When comparisons were made to Floyd et al.-prepared poliovirus (56) which had not been previously exposed to monochloramine, a development of resistance of the 8 times-exposed poliovirus was quite evident. The 8 times-exposed poliovirus was exposed to 8.95 mg/L of monochloramine, and was 2.3 times more resistant to monochloramine than either the unexposed Floyd et al.-prepared virus (56) or the virus exposed 7 times to monochloramine in study 8. A gradual progression in the development of virus resistance was evident in the research with the Floyd et al. (56) inocula. Perhaps if we had carried out the studies further and exposed the regrown virus several more times to monochloramine more consistant resistance patterns could have emerged.

DISINFECTION OF HEp-2 CELL-ASSOCIATED POLIOVIRUS 1 WITH HYPOCHLOROUS ACID AND MONOCHLORAMINE

Disinfection studies with animal cell-associated poliovirus 1 were performed using two continuous cell lines, Human Epidermoid Carcinoma (HEp-2) and Buffalo Green Monkey (BGM) kidney cells. The cell-associated virus system approximates the state of viruses as they are excreted from the body into domestic sewage. Wastewater however contains many organic substances which consume free chlorine. On the other hand, chlorine which has combined with ammonia to form chloramines can be an important factor in disinfection. Thus, these studies were performed to ascertain the effects on disinfection rates of HEp-2 and BGM cell-associated poliovirus 1, which also provided the turbidity to the systems. The first study described below was with HEp-2 cell-associated poliovirus.

Disinfection of HEp-2 cell-associated poliovirus 1 with 2.2 mg/L and 2.28 mg/L hypochlorous acid at 5C and pH 6 is shown in Figure 23. This HEp-2 cell-associated poliovirus 1 preparation showed protection of virions when compared to the aggregated preparation of poliovirus 1 (See Section 4, Materials and Methods for preparation of cell-associated viruses and virus aggre-

TABLE 4. Results of Repetitive Exposures to Monochloramines of Bates et al.-Prepared Poliovirus 1 (22) at 15C and pH 9

Exposures to NH_2Cl	0	0	1	2	3	4	5	6	7	8	7,8[a]
Study Number	10	1	2	3	4	5	6	7	8	9	10
Date of Study	8/29	4/1	4/18	5/2	5/9	7/8	7/18	7/25	8/8	8/15	8/29
Initial Titer (PFU/ml)	$1.6x10^5$	$2.5x10^5$	$4.1x10^4$	$3.2x10^4$	$1.4x10^3$	$9.4x10^4$	$6.1x10^4$	$5.2x10^3$	$3.5x10^3$	$2.7x10^3$	$8.9x10^2$
Time[b]	Percent Survival										
1	162	80.0	62.5	50.0	25.7	54.2	TNTC[c]	TNTC	42.9	72.7	135
10	-[d]	-	-	-	-	-	31.1	-	21.7	-	-
15	106	-	-	-	-	10.4	-	18.0	-	24.7	82.0
20	-	-	-	-	-	-	14.9	-	28	-	-
30	27.5	TNTC	1.6	0.41	ND[e]	2.5	2.5	2.1	7.7	7.1	28.1
45	35.0	-	-	-	-	0.49	1.5	0.15	0.17	9.1	22.5
60	17.5	TNTC	0.05	0.028	ND	0.21	0.05	0.05	0.037	2.5	15.7
75	6.9	-	-	-	-	-	ND	0.08	ND	1.1	6.3
90	TNTC	-	-	-	ND	0.04	0.07	ND	ND	0.29	5.5
105	-	-	-	-	-	-	0.01	ND	ND	0.24	-
120	TNTC	TNTC	0.014	ND	ND	0.004	0.003	-	ND	0.19	3.9
180	TNTC	1.2	0.003	ND	ND	ND	ND	ND	ND	ND	0.45
240	0.36	-	-	ND	ND	ND	ND	ND	ND	ND	0.3
Final NH_2Cl(mg/L)	9.6	12.0	11.2	12.2	11.1	11.3	13.1	11.8	10.15	10.2	9.3

a Viruses exposed 7 and 8 times were combined as the inoculum.
b In minutes after initial exposure.
c Too numerous to count.
d Not sampled.
e Not detected, no recovery.

TABLE 5. Results of Repetitive Exposures to Monochloramine of Floyd et al.-Prepared Poliovirus 1 (56) at 15C and pH 9

Exposures to NH_2Cl	0	0	1	2	3	4	5	6	7	8	8
Study Number	10	1	2	3	4	5	6	7	8	9	10
Date of Study	8/21	4/1	4/18	5/2	5/9	7/8	7/18	7/25	8/8	8/15	8/21
Initial Titer (PFU/ml)	$9.9x10^3$	$1.0x10^5$	$5.6x10^3$	$7.3x10^2$	$4.5x10^2$	$2.4x10^5$	$1.2x10^6$	$7.1x10^4$	$4.6x10^3$	$1.6x10^4$	$1.2x10^4$
Time[a]	Percent Survival										
1	94.9	26	112	61.6	4.7	TNTC[b]	TNTC	46.5	89.1	76	104
10	-[c]	-	-	-	-	-	TNTC	-	11.5	-	-
15	23.2	-	-	-	0.22	TNTC	-	8.6	-	25	45.8
20	-	-	-	-	-	-	TNTC	-	3.9	-	-
30	2.2	0.75	0.3	0.41	ND[d]	15	TNTC	2.0	2.4	5.7	18.3
45	0.66	-	-	-	-	5	TNTC	0.04	0.43	6	4.9
60	0.52	0.24	ND	0.14	ND	2.1	0.11	0.04	0.46	4.3	2.3
75	0.2	-	-	-	-	-	ND	-	0.22	1.4	0.89
90	0.1	-	-	-	ND	0.74	ND	0.002	0.115	0.37	-
105	-	-	-	-	-	-	ND	-	0.029	0.36	-
120	0.05	0.003	0.18	ND	ND	0.12	ND	ND	0.059	0.14	0.28
180	ND	ND	ND	ND	ND	0.008	ND	ND	ND	ND	0.011
240	ND	-	ND	ND	-	0.002	0.003	0.002	ND	-	ND
Final NH_2Cl (mg/L)	9.15	12.1	11.4	12.3	11.1	11.75	12.8	12.1	10.15	10.4	8.95

a In minutes after initial exposure.
b Too numerous to count.
c Not sampled.
d Not detected, no recovery.

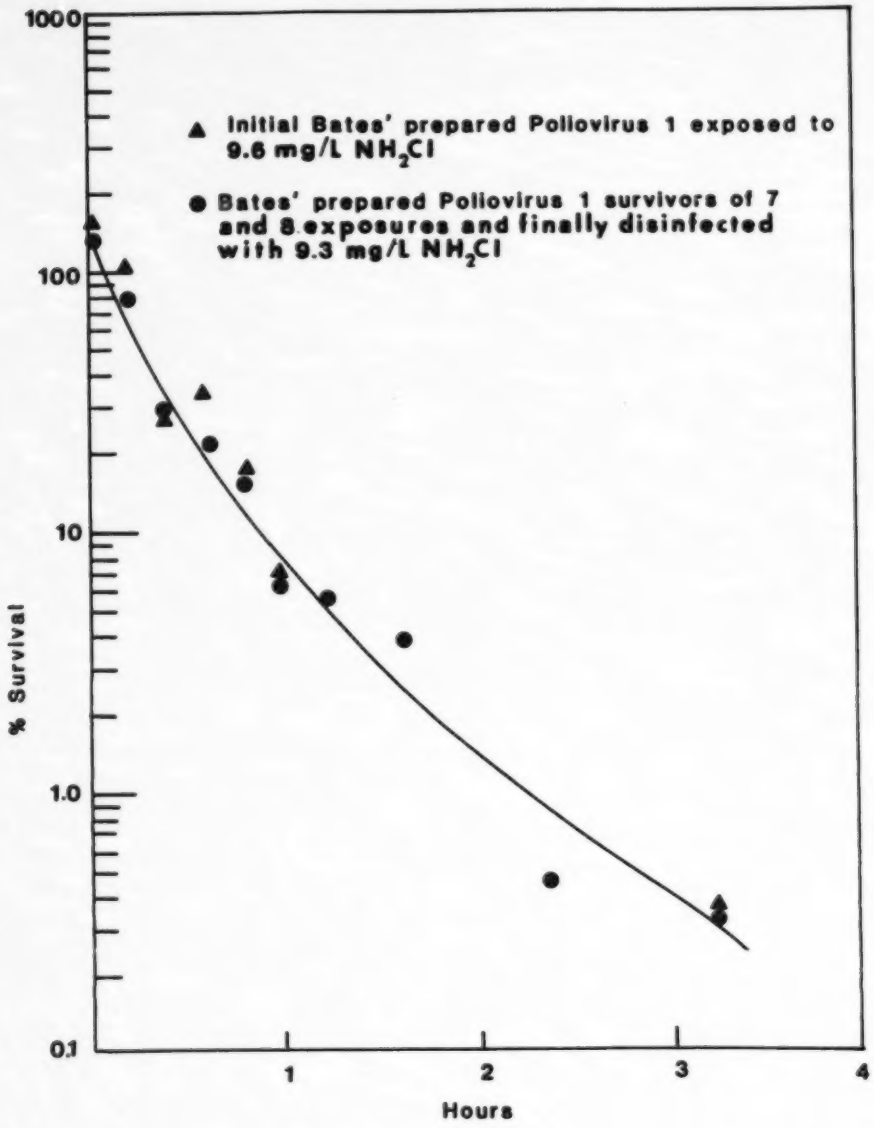

Figure 21. Inactivation of Bates et al.-prepared poliovirus 1 (22) at pH 9 and 15C before and after repeated exposure to monochloramine.

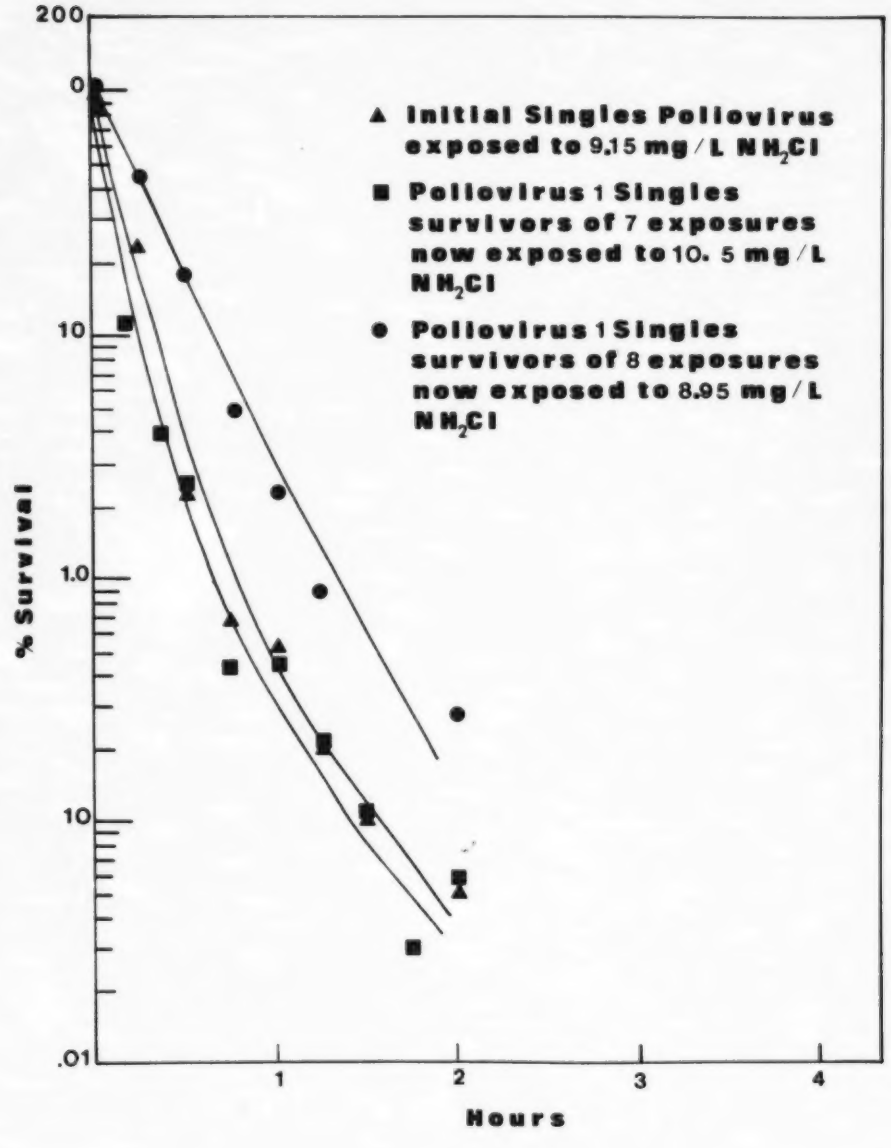

Figure 22. Inactivation of Floyd et al.-prepared poliovirus 1 (56) at pH 9 and 15C before and after repeated exposure to monochloramine

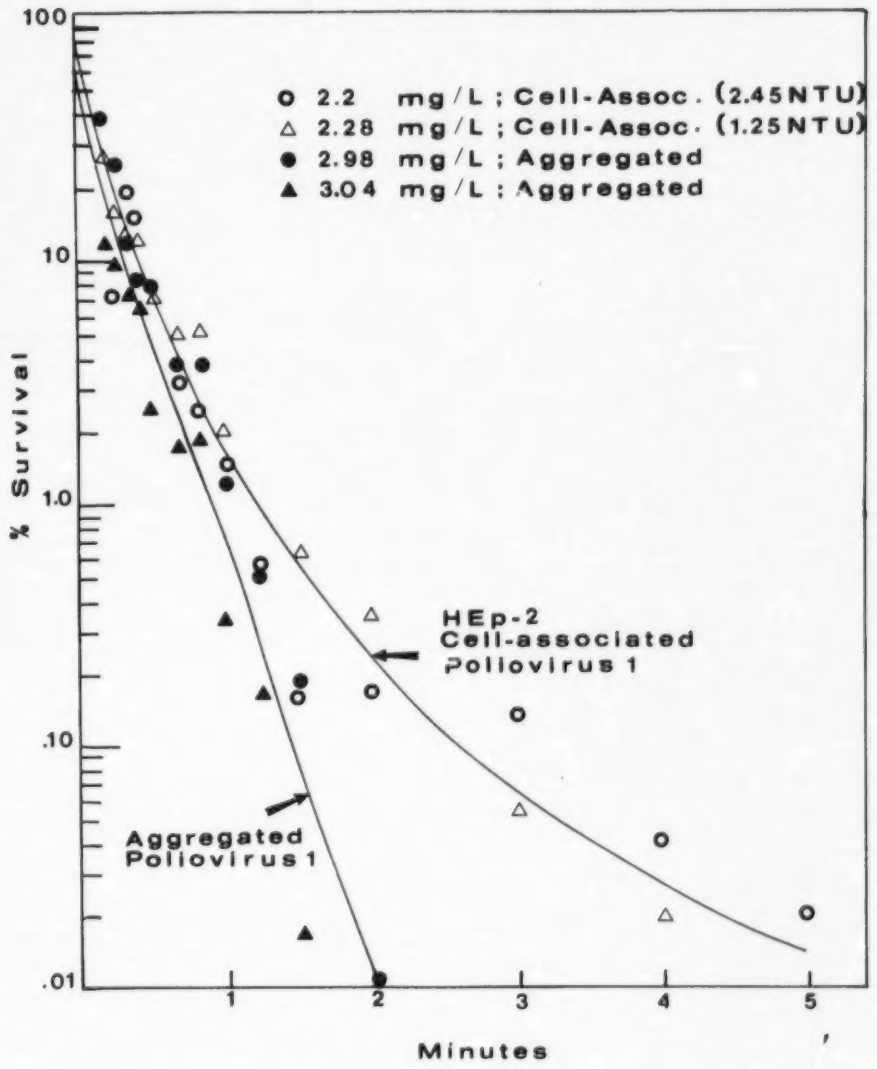

Figure 23. Inactivation of aggregated and HEp-2 cell-associated poliovirus 1 with hypochlorous acid at pH 6 and 5C

gates). This effect is most dramatically demonstrated at the 99.9% through 99.99% inactivation portion of the curves. Percent survival data including final turbidity levels in nephelometric turbidity units (NTU), final hypochlorous acid levels and test dates are contained in Table 6. An earlier study by Hoff (23) on the relationship of turbidity to disinfection of potable water using HEp-2 cell-associated poliovirus showed a similar interference with hypochlorous acid inactivation of the cell-associated viruses.

Comparison of the disinfection at 5C of HEp-2 cell-associated poliovirus 1 with monochloramine at pH 9 and hypochlorous acid at pH 6 is shown in Figure 24. As can be seen from the graph, 3.04 mg/L hypochlorous acid inactivated 99% of the HEp-2 cell-associated virions in 3 minutes, while 12.2 mg/L monochloramine inactivated 99% in 120 minutes, making the hypochlorous acid 40 times more effective as a disinfectant than the monochloramine under these test conditions.

Studies on the effect of organic turbidity on the disinfection of HEp-2 cell-associated poliovirus with monochloramine concentrations ranging from 4.15 to 21.0 mg/L at 5C and pH 7 and 9 are shown in Figure 25. This is a concentration-time plot at the 90% death point which also shows the inactivation of poliovirus 1 singles with monochloramine at 5C and pH 9. Increasing the monochloramine dosage almost twice from 12.2 to 21.0 mg/L at pH 9 in the presence of almost the same turbidity reduced the time required for 90% virus inactivation, i.e., from 50 minutes at 12.2 mg/L to 30 minutes at 21.0 mg/L monochloramine. Increasing the turbidity in Figure 25 from 0.8 NTU to 2.0 NTU at almost the same monochloramine levels in two cases (10.35 and 11.3 mg/L, respectively) at pH 7 significantly decreased disinfection efficiency. Turbidity caused by the presence of animal cells interfered with the disinfection process. The pH change from 9 to 7 had no apparent effect on the rate of inactivation of poliovirus 1, whether or not the virus was associated with animal cells or not.

Some tests on HEp-2 cell-associated poliovirus 1 were run concurrently with Mr. Donald Berman of the U.S. Environmental Protection Agency, using 0.05 M phosphate buffer at 5C and pH 7. Table 7 gives for these studies the % survival data, final turbidity levels in Nephelometric Turbidity Units (NTU), initial and final monochloramine levels, and test dates. The same amount of HEp-2 cell-associated poliovirus 1 inoculum per unit volume of buffer was added for each test. As seen in Table 7, poliovirus 1 singles were used as positive virus controls on test dates 10/31/80 and 11/14/80. By inspection of the table, it can be seen that the poliovirus 1 singles inactivation rates are more rapid at the end of the exposure time period. Some protection of poliovirus was occurring due to the association with HEp-2 tissue culture cells.

HEp-2 cell-associated viruses have been also used by Foster et al. (89) and Emerson et al. (90) in their studies with ozone inactivation of cell-associated viruses (both poliovirus and coxsackievirus). Although the disinfectant was different, their results indicated again the protective effect of virus-association with HEp-2 cells. Their inactivation data on poliovirus and coxsackievirus indicated that cell-associated viruses required higher ozone residuals for inactivation than unassociated viruses. They emphasized

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TABLE 6. Disinfection of HEp-2 Cell-Associated Poliovirus 1 with Hypochlorous Acid at 5C and pH 6

	HEp-2 Cell-Associated Poliovirus 1		Poliovirus 1 Control (Aggregated)	
Date of Test:	7/12/79	7/26/79	7/12/79	7/26/79
Concentration of HOCl in mg/L:				
Initial:	3.08	3.04	3.08	3.04
Final:	2.28	2.20	3.04	2.98
NTU[a]:	1.25	2.45	0.51	0.45
Control Titer:	1.05×10^5	7.55×10^4	6.05×10^4	4.85×10^4

Time	PFU/ml	% Survival	PFU/ml	% Survival	PFU/ml	% Survival	PFU/ml	% Survival
10 Sec.	2.8×10^4	26.7	2.2×10^4	29.1	7.2×10^3	11.9	1.9×10^4	39.2
15 "	1.7×10^4	16.1	5.4×10^3	7.2	5.7×10^3	9.4	1.2×10^4	24.7
20 "	1.4×10^4	13.2	1.5×10^4	19.9	4.4×10^3	7.3	6.0×10^3	12.4
25 "	1.3×10^4	12.4	1.1×10^4	14.6	4.0×10^3	6.6	4.1×10^3	8.45
30 "	7.5×10^3	7.1	5.8×10^3	7.7	1.5×10^3	2.5	3.8×10^3	7.8
40 "	5.3×10^3	5.1	2.5×10^3	3.3	1.0×10^3	1.7	1.9×10^3	3.9
50 "	5.4×10^3	5.1	2.0×10^3	2.6	1.1×10^2	1.8	1.8×10^2	3.7
60 "	2.1×10^3	2.0	1.15×10^3	1.5	2.0×10^2	0.33	6.2×10^2	1.28
75 "	1.7×10^3	1.6	4.3×10^2	0.57	1.0×10^2	0.165	2.7×10^1	0.56
90 "	6.6×10^2	0.63	1.2×10^2	0.16	1.0×10^1	0.0165	9.4×10^0	0.19
2 Min.	1.9×10^2	0.35	1.3×10^2	0.167	ND	ND	2.0×10^0	0.004
3 "	2.9×10^1	0.055	1.0×10^2	0.135	ND	ND	ND	ND
4 "	1.0×10^1	0.020	3.2×10^1	0.04	ND	ND	ND	ND
5 "	3.0×10^0	0.006	1.6×10^1	0.02	ND	ND	ND	ND
10 "	ND[b]	ND	2.0×10^0	0.003				
20 "	ND	ND	ND	ND				
30 "	ND	ND	ND	ND				
40 "	ND	ND	ND	ND				
50 "	ND	ND	ND	ND				
60 "	ND	ND	2.0×10^0	0.003				

a Nephelometric Turbidity Units (NTU)
b Not detected.

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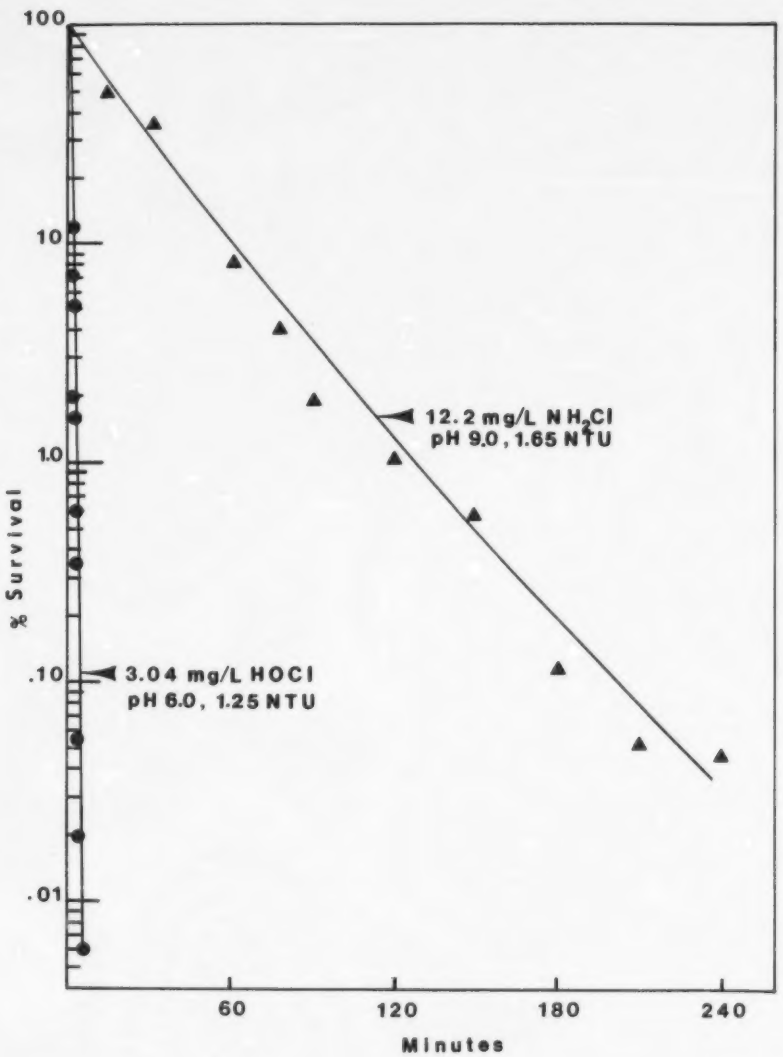

Figure 24. Inactivation of HEp-2 cell-associated poliovirus 1 at 5C by hypochlorous acid at pH 6 and monochloramine at pH 9

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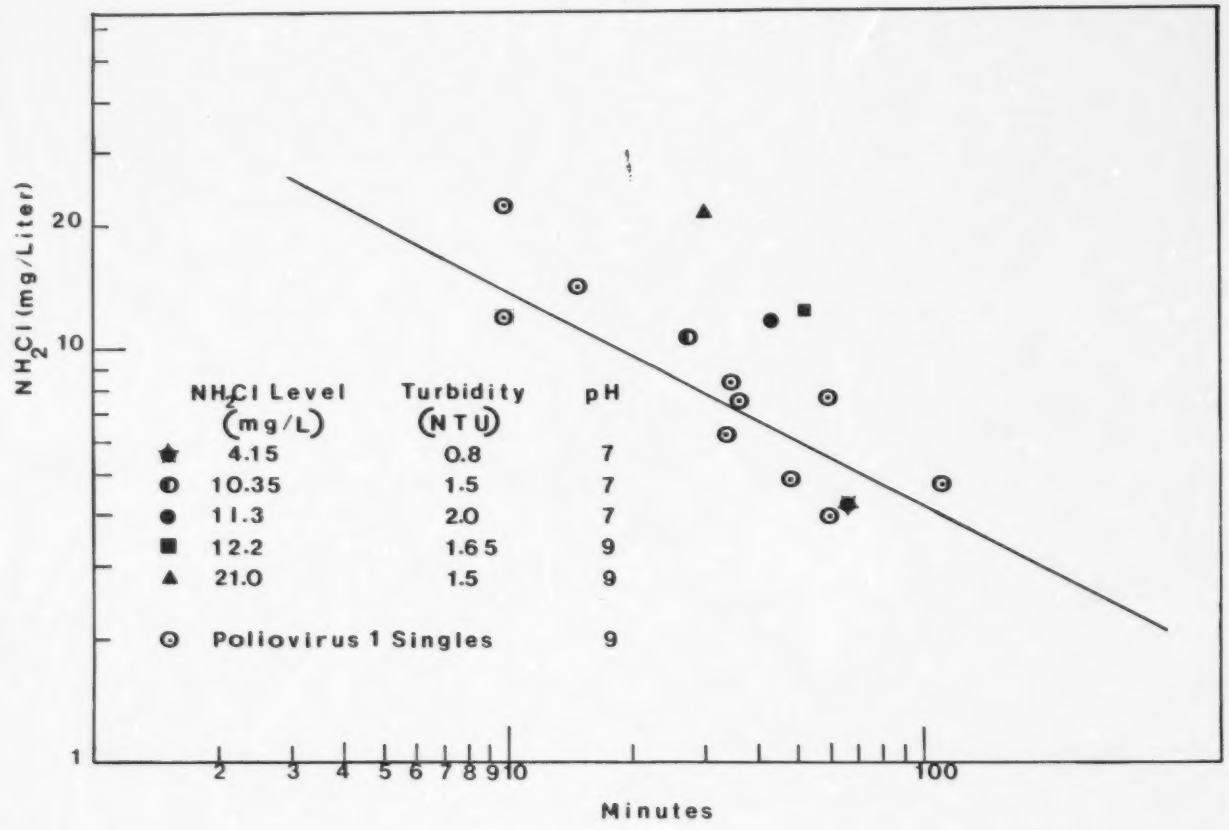

Figure 25. Concentration-time relationship for 90% inactivation of poliovirus 1 singles and HEp-2 cell-associated poliovirus 1 at different turbidity levels and concentrations of monochloramine at 5C and pH 7 and 9

TABLE 7. Disinfection of HEp-2 Cell-Associated Poliovirus 1 and Poliovirus 1 Singles with Monochloramine at 5C and pH 7

	HEp-2 Cell-Associated			Poliovirus 1 Singles	
Date of Test:	10/24/80	10/31/80	11/14/80	10/31/80	11/14/80
Concentration of NH_2Cl in mg/L					
Initial:	4.30	11.5	10.6	11.5	10.6
Final:	4.5	11.3	10.35	11.5	10.55
NTU[a]:	0.8	2.0	1.5	0.2	b
Control Titer:	$4.6x10^5$	$4.6x10^5$	$9.8x10^5$	$1.85x10^4$	$2.3x10^4$

Time	PFU/ml	% Survival	PFU/ml	% Survival	PFU/ml	% Survival	PFU/ml	% Survival	PFU/ml	% Survival
1 Min.	$4.55x10^5$	98.9	$6.9x10^5$	150.0	$1.3x10^5$	13.3	$9.0x10^3$	48.6	$1.65x10^4$	71.7
2 "	$3.2x10^5$	69.6	$4.0x10^5$	87.0	$5.0x10^4$	51	$1.3x10^4$	70.3	$9.4x10^3$	40.9
5 "	$1.8x10^5$	39.1	$1.2x10^5$	26.0	$6.0x10^4$	6.1	$2.1x0^4$	113.5	$1.1x10^4$	47.8
10 "	$7.1x10^4$	15.4	$6.0x10^5$	13	$1.6x10^5$	16.3	$1.3x10^4$	70.3	$6.0x10^3$	26.1
15 "	$1.2x10^5$	26.0	$1.5x10^5$	32.6	$1.9x10^5$	19.4	$1.3x10^4$	70.3	$5.0x10^3$	21.7
20 "	$1.2x10^5$	26.0	$8.6x10^4$	18.7	$1.6x10^5$	16.3	$9.5x10^3$	51.4	$2.8x10^3$	12.2
30 "	$4.6x10^4$	10.0	$8.7x10^4$	18.9	$9.3x10^4$	9.5	$3.6x10^3$	19.5	$1.0x10^3$	4.8
40 "	$4.4x10^4$	9.6	$3.4x10^4$	7.4	$6.5x10^4$	6.6	$1.65x10^3$	8.9	$7.2x10^2$	3.1
50 "	$5.9x10^4$	12.8	$7.3x10^4$	15.9	$3.1x10^4$	3.2	$9.0x10^2$	4.9	$3.9x10^2$	1.7
60 "	$5.3x10^4$ c	11.5	$1.9x10^4$	4.1	$4.7x10^4$	4.8	b	b	b	b
75 "	$4.0x10^4$ c	8.7^c	$2.1x10^4$	4.6	$4.0x10^4$	4.1	$2.3x10^2$	1.2	$1.7x10^2$	0.76
90 "	$4.0x10^3$ c	0.87^c	$1.6x10^4$	3.5	$2.0x10^4$	2.0	$1.7x10^2$	0.92	$9.7x10^1$	0.42
120 "	$4.0x10^3$ c	0.87^c	$1.9x10^4$	4.1	b	b	$4.7x10^1$	0.25	b	b
130 "	b	b	b	b	$1.7x10^4$	1.7	b	b	$2.7x10^1$	0.13
150 "	b	b	b	b	$7.8x10^3$	0.79	b	b	b	b
180 "	$4.0x10^3$ c	0.87^c	$8.4x10^3$	1.8	$1.9x10^3$	0.27	$2.5x10^1$	0.14	$2.5x10^1$	0.11

a Nephelometric turbidity units
b Not done
c Estimated value

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that since the HEp-2 cells were approximately 10 to 15 μm in size, such sized particles could be easily removed from finished water by filtration (89). Similar sized particles are also found in wastewater effluent after activated sludge treatment.

DISINFECTION OF BGM CELL-ASSOCIATED POLIOVIRUS 1 WITH HYPOCHLOROUS ACID, MONOCHLORAMINE, AND DICHLORAMINE

The inactivation of Buffalo Green Monkey (BGM) cell-associated poliovirus 1 was studied using three disinfectants, i.e. hypochlorous acid (HOCl) at 15C and pH 6.0, monochloramine (NH_2Cl) at 15C and pH 9, and dichloramine at 5C and pH 4.5. Figure 26 is a comparison of the disinfection at 15C and pH 6 of BGM cell-associated poliovirus 1 by 0.03, 0.42, and 1.20 mg/L hypochlorous acid at turbidity levels of 1.75, 1.05, and 1.10 NTU, respectively. All the survival curves showed extended tailings caused by the association of the poliovirus 1 to the cells and to themselves (aggregation) during the disinfection process. A comparison between the disinfection of BGM cell-associated poliovirus 1 by monochloramine and hypochlorous acid at similar turbidities and concentrations is shown in Figure 27. Whereas 90% of the cell-associated viruses were inactivated in 15 seconds by the hypochlorous acid, 95 minutes was required to reach the 90% inactivation point with the monochloramine. Even under conditions as difficult as this to disinfect viruses, hypochlorous acid was about 380 times as effective as the monochloramine. Figure 28 is a summary monochloramine concentration-time plot for the 90% inactivation of BGM cell-associated and unassociated viruses. The BGM cell-associated poliovirus 1 points are represented by bold symbols. Most of these "associated" points were above the poliovirus 1 unassociated singles curve, indicating that the cell-associated viruses were being protected from inactivation. In another study by us of the disinfection of BGM cell-associated poliovirus 1 by chlorine dioxide we were unable to demonstrate such protection (64).

A final study was done (see Figure 29) comparing the inactivation of BGM cell-associated polioviruses to the survival of unassociated poliovirus 1 singles, using dichloramine as the disinfectant at 5C and pH 4.5. No differences were observed in the rates of disinfection between the two poliovirus preparations, although the dichloramine concentrations were similar (17.0 mg/L for the unassociated versus 17.35 mg/L for the associated poliovirus). The lack of protection could be due to rapid penetration of the cell mass by the dichloramine.

COLIFORM DISINFECTION STUDIES

Disinfection studies using coliforms were divided into two groups: (a) disinfection of naturally-occurring coliforms from fecal suspensions, and (b) disinfection of fecal coliforms associated with primary effluent solids.

Disinfection with Hypochlorous Acid of Naturally-Occurring Coliforms Obtained From Human Feces

Fecal suspensions were prepared as outlined in Preparation of Fecal Particulates in Section 4 of this report. These fecal suspensions provided

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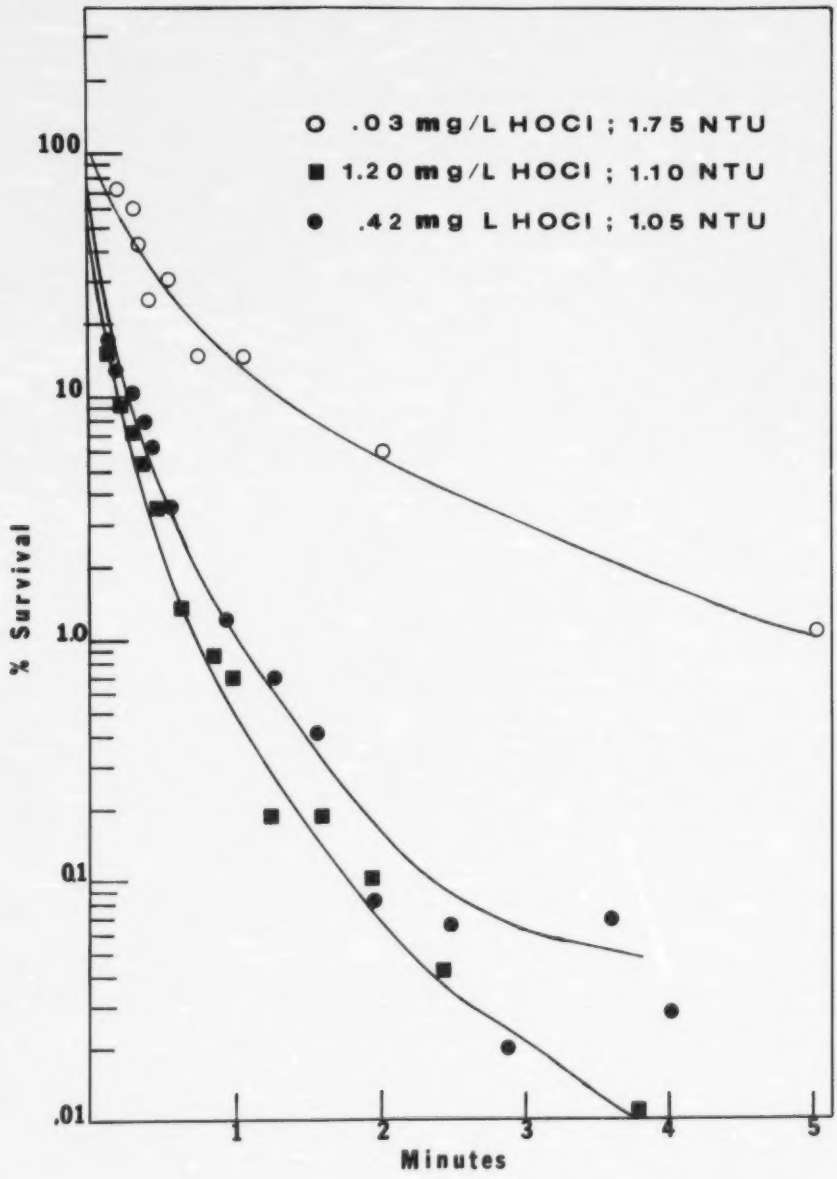

Figure 26. Inactivation of BGM cell-associated poliovirus 1 by hypochlorous acid at 15C and pH 6

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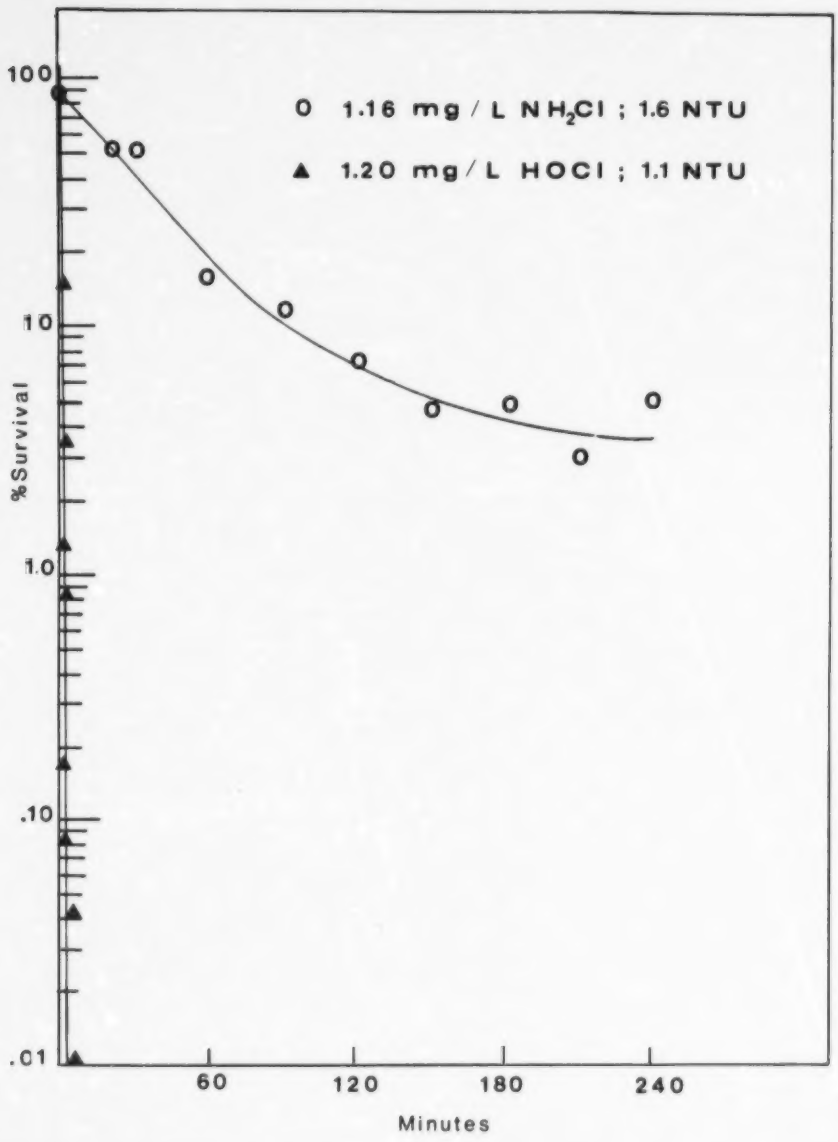

Figure 27. Inactivation of BGM cell-associated poliovirus 1 by monochloramine (pH 9) and hypochlorous acid (pH 6) at 15C

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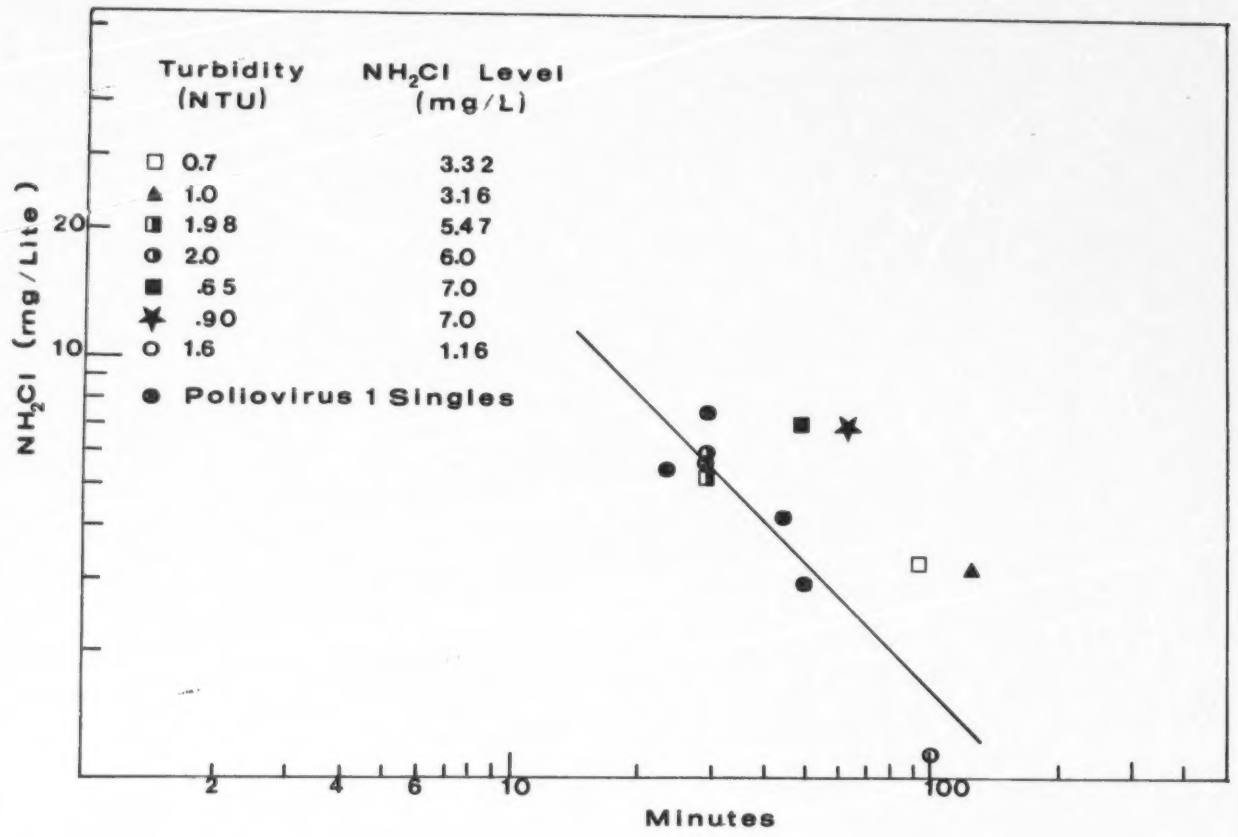

Figure 28. Concentration-time relationship for 90% inactivation of BGM cell-associated and unassociated poliovirus 1 by various concentrations of monochloramine at 15C and pH 9

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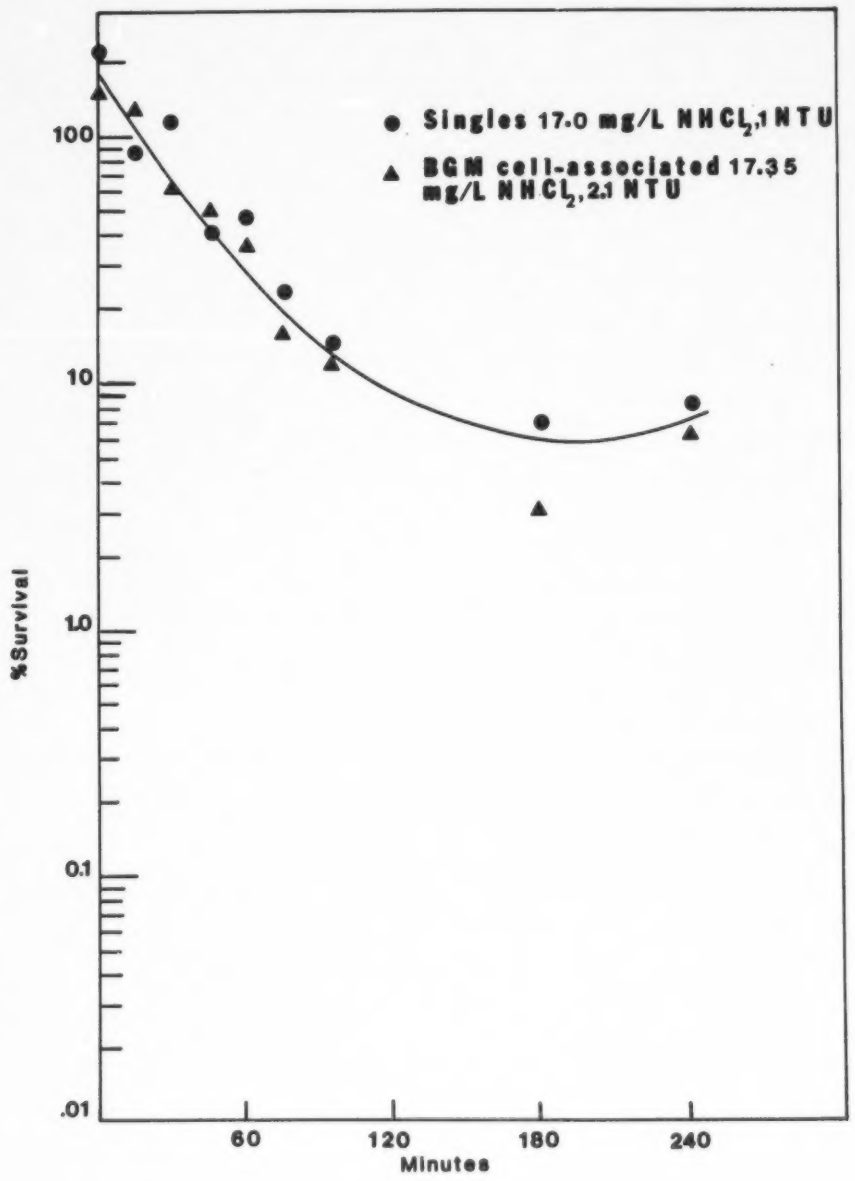

Figure 29. Inactivation of BGM cell-associated poliovirus and poliovirus 1 singles by dichloramine at pH 4.5 and 5C

an uncontested source of naturally-occurring coliforms which had not been nurtured by laboratory media. Difficulties soon arose due to the chlorine demand of the fecal suspensions. Repeated washings (10 times) of the fecal suspension did not remove the chlorine demand or result in a suspension containing a consistant chlorine demand. Table 8 shows the shifting levels of chlorine demand encountered, although the chlorine demand was found to be sometimes lower at 5C than 20C. Therefore, it was decided to do further studies at 5C. Figure 30 shows a typical hypochlorous acid disinfection study at 5C and pH 6 with naturally occurring coliforms at a turbidity of 3.2 NTU. In similar studies done by Foster et al. (89), no protection of fecal-associated coliforms was evident at 1.0 NTU, although some protection was shown with a turbidity of 5.0 NTU and an inital ozone level of 0.10 mg/L and below.

Disinfection With Monochloramine of Naturally-Occurring Coliforms Obtained From Primary Effluent

Laboratory studies were performed on naturally-occurring total coliforms obtained from primary effluent. These coliforms were associated with particles which, when suspended in 0.05 M phosphate buffer, gave a turbidity level of 5.0 NTU. Table 9 is a summary of the test levels of monochloramine used and their corresponding turbidity levels. Survival data for the naturally-occurring coliforms after their exposure to different monochloramine levels at different turbidities (Table 9) are recorded in Table 10. Coliforms were present after 60 minutes contact time in tests 2 and 3, at initial monochloramine levels of 12.2 and 5.1 mg/L, respectively. The turbidity levels were similar in these two tests (5.8 NTU in test 2, 5.5 NTU in test 3). Tests 1, 4, and 5 at initial monochloramine levels of 23.2, 10.3, and 10.3, respectively, showed a more rapid coliform decline after 1 minute in test 1, and after 10 minutes in tests 4 and 5. In tests 1, 4, and 5 coliforms were detected erratically. For instance, coliforms were found after 105 minutes in tests 1 and 5, although substantial kill had occurred after one minute and 10 minutes, respectively. Coliforms were present after 120 minutes contact time with monochloramine during test 3, which contained the lowest level of monochloramine (4.95 mg/L NH_2Cl) used. These results with naturally-occurring coliforms obtained from primary effluent were substantially consistent to those of Hoff (23), who used 0.5 mg/L hypochlorous acid and turbidities of 1 and 5 NTU. His survival curves extended over a 60 minute period at both 1 and 5 NTU's. The results reported here were similar in that the organic material from the primary effluent associated with the coliforms and particles demonstrated a protective effect in tests 2 and 3, and to a more limited extent in tests 4 and 5.

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TABLE 8. Chlorine Demand of Suspensions of Naturally-Occurring Coliforms after a 30 Minute Exposure

Initial HOCl Level (mg/L)	Final HOCl Level (mg/L)	Chlorine Demand of 1.0 ml of Suspension (mg/L)	Temperature (C)	NTU[a]
0.91	0.34	0.57	20	-[b]
0.66	0.27	0.39	5	3.0
0.7i	0.27	0.43	5	3.2
0.74	0.16	0.58	20	1.75
0.50	0.11	0.40	5	1.75
0.74	0.32	0.42	5	1.75
1.05	0.33	0.72	20	2.2
0.75	0.25	0.50	5	2.2
0.85	0.30	0.55	5	2.0
1.05	0.51	0.54	20	-
1.05	0.55	0.50	20	-
1.05	0.66	0.39	5	-
1.05	0.61	0.44	5	-
0.66	0.42	0.24	5	2.0
0.74	0.64	0.10	5	2.0
2.65	0.14	2.51	5	16
3.35	0.10	3.25	5	18

[a] Nephelometric Turbidity Units.
[b] Not done.

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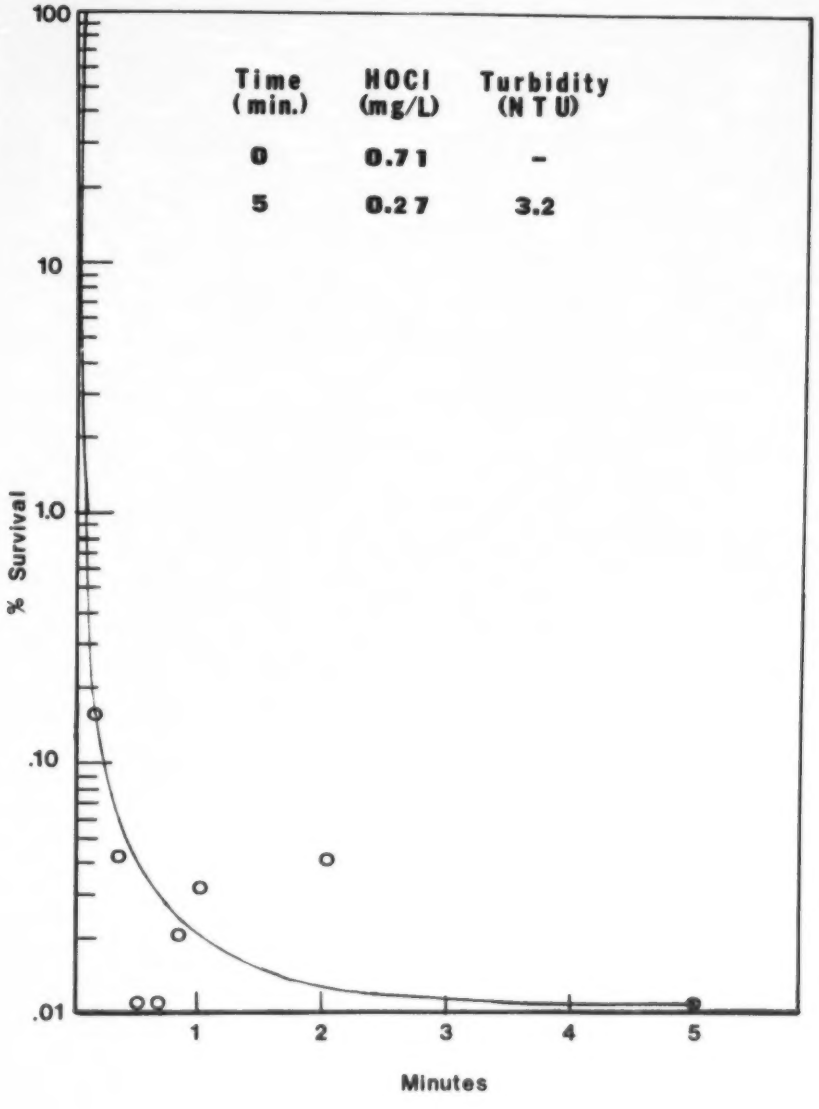

Figure 30. Inactivation of naturally-occurring coliforms by hypochlorous acid at 5C and pH 6

TABLE 9. Monochloramine and Turbidity Levels Used For Disinfection at 5C and pH 7 of Naturally-Occurring Coliforms Associated with Primary Effluent Solids

	Test number				
	1	2	3	4	5
Monochloramine (NH_2Cl) Levels					
Initial NH_2Cl (mg/L)	23.2	12.2	5.1	10.3	10.3
Final NH_2Cl (mg/L)	22.6	11.95	4.95	9.9	9.95
Turbidity (NTU) Concentrations					
NTU Test	1.8[a]	5.8	5.5	8.0 to 1.75[b]	5.5

[a] Turbidity level run at 5C, the remaining turbidity measurements were done at 25C.

[b] Flocculation in test beaker made turbidity reading fluctuate.

TABLE 10. The Inactivation by Monochloramine of Naturally-Occurring Coliforms Associated with Primary Effluent Solids at 5C and pH 7

	Test number				
	1	2	3	4	5
	Most Probable Number/100 ml				
Initial	5.4×10^5	1.8×10^5	5.4×10^5	9.2×10^5	3.5×10^5
Final	3.6×10^4	6.3×10^4	5.4×10^5	7.9×10^5	3.5×10^5
Average	2.9×10^5	1.2×10^5	5.4×10^5	8.5×10^5	3.5×10^5
Test times (minutes)					
1	1.4×10^5	1.8×10^5	5.4×10^5	5.4×10^5	1.6×10^6
10	ND[b]	1.3×10^2	1.6×10^5	1.6×10^3	2.8×10^3
20	ND	13	2.4×10^4	ND	2
30	ND	1.3×10^2	5.4×10^3	ND	5
45	ND	23	1.6×10^3	2	ND
60	2	23	17	ND	ND
75	ND	ND	27	2	2
90	ND	ND	2	ND	ND
105	2	ND	2	ND	2
120	ND	ND	2	ND	ND
180	-[c]	-	ND	-	-

a Analysis of coliforms through confirmed most probable number test procedure.
b Not detected, no recovery.
c Not sampled.

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